Differential effects of peripheral and brain tumor necrosis factor on inflammation, sickness, emotional behavior and memory in mice

Klaus, Federica ; Paterna, Jean-Charles ; Marzorati, Elisa ; Sigrist, Hannes ; Götze, Lea ; Schwendener, Severin ; Bergamini, Giorgio ; Jehli, Elisabeth ; Azzinnari, Damiano ; Fuertig, René ; Fontana, Adriano ; Seifritz, Erich ; Pryce, Christopher R

Abstract: Tumor necrosis factor alpha (TNF) is increased in depression and clinical-trial evidence indicates that blocking peripheral TNF has some antidepressant efficacy. In rodents, peripheral or intracerebroventricular TNF results in sickness e.g. reduced body weight, altered emotional behavior and impaired memory. However, the underlying pathways and responsible brain regions are poorly understood. The aim of this mouse study was to increase understanding by comparing the effects of sustained increases in TNF in the circulation, in brain regions impacted by increased circulating TNF, or specific brain regions. Increased peripheral TNF achieved by repeated daily injection (IP-TNF) or osmotic pump resulted in decreased body weight, decreased saccharin (reward) consumption, and increased memory of an aversive conditioned stimulus. These effects co-occurred with increased plasma interleukin-6 and increased IP-derived TNF in brain peri-ventricular regions. An adenovirus-associated viral TNF vector (AAV-TNF) was constructed, brain injection of which resulted in dose-dependent, sustained and region-specific TNF expression, and was without effect on blood cytokine levels. Lateral ventricle AAV-TNF yielded increased TNF in the same brain regions as IP-TNF. In contrast to IP-TNF it was without effect on body weight, saccharin consumption and fear memory, although it did increase anxiety. Hippocampal AAV-TNF led to decreased body weight. It increased conditioning to but not subsequent memory of an aversive context, suggesting impaired consolidation; it also increased anxiety. Amygdala AAV-TNF was without effect on body weight and aversive stimulus learning-memory, but reduced saccharin consumption and increased anxiety. This study adds significantly to the evidence that both peripheral and brain region-specific increases in TNF lead to both sickness and depression- and anxiety disorder-relevant behavior and do so via different pathways. It thereby highlights the complexity in terms of indirect and direct pathways via which increased TNF can act and which need to be taken into account when considering it as a therapeutic target.

DOI: https://doi.org/10.1016/j.bbi.2016.08.001

Originally published at:
Klaus, Federica; Paterna, Jean-Charles; Marzorati, Elisa; Sigrist, Hannes; Götze, Lea; Schwendener, Severin; Bergamini, Giorgio; Jehli, Elisabeth; Azzinnari, Damiano; Fuertig, René; Fontana, Adriano; Seifritz, Erich; Pryce, Christopher R (2016). Differential effects of peripheral and brain tumor necrosis factor on inflammation, sickness, emotional behavior and memory in mice. Brain, Behavior, and Immunity, 58:310-326.
DOI: https://doi.org/10.1016/j.bbi.2016.08.001

Differential effects of peripheral and brain tumor necrosis factor on inflammation, sickness, emotional behavior and memory in mice

Federica Klaus[1,5,6], Jean-Charles Paterna[2,] , Elisa Marzorati[1], Hannes Sigrist[1], Lea Götze[1], Severin Schwendener, Giorgio Bergamini[1,6], Elisabeth Jehli[1], Damiano Azzinnari[1,6], René Fuertig[3], Adriano Fontana[4], Erich Seifritz[5,6], Christopher R. Pryce[1,6,*]

[1] Preclinical Laboratory for Translational Research into Affective Disorders, Department of Psychiatry, Psychotherapy and Psychosomatics, Psychiatric Hospital, University of Zurich, Switzerland

[2] Viral Vector Facility, Neuroscience Center Zurich, University of Zurich and ETH Zurich, Switzerland

[3] CNS Diseases Research Germany, Boehringer Ingelheim Pharma GmbH & Co. KG., Biberach, Germany

[4] Institute of Experimental Immunology, Inflammation and Sickness Behaviour, University of Zurich, Switzerland

[5] Department of Psychiatry, Psychotherapy and Psychosomatics, Psychiatric Hospital, University of Zurich, Switzerland

[6] Neuroscience Center Zurich, University of Zurich and ETH Zurich, Switzerland

* Corresponding author: PLaTRAD, Department of Psychiatry, Psychotherapy & Psychosomatics, Psychiatric Hospital, University of Zurich, August Forel-Strasse 7, CH-8008 Zürich, Switzerland. E-mail: christopher.pryce@bli.uzh.ch

Abstract

Tumor necrosis factor alpha (TNF) is increased in depression and clinical-trial evidence indicates that blocking peripheral TNF has some antidepressant efficacy. In rodents, peripheral or intracerebroventricular TNF results in sickness e.g. reduced body weight, altered emotional behavior and impaired memory. However, the underlying pathways and responsible brain regions are poorly understood. The aim of this mouse study was to increase understanding by comparing the effects of sustained increases in TNF in the circulation, in brain regions impacted by increased circulating TNF, or specific brain regions. Increased peripheral TNF achieved by repeated daily injection (IP-TNF) or osmotic pump resulted in decreased body weight, decreased saccharin (reward) consumption, and increased memory of an aversive conditioned. These effects co-occurred with increased plasma interleukin-6 and increased IP-derived TNF in brain peri-ventricular regions. An adenovirus-associated viral TNF vector (AAV-TNF) was constructed, brain injection of which resulted in dose-dependent, sustained and region-specific TNF expression, and was without effect on blood cytokine levels. Lateral ventricle AAV-TNF yielded increased TNF in the same brain regions as IP-TNF. In contrast to IP-TNF it was without effect on body weight, saccharin consumption and fear memory, although it did increase anxiety. Hippocampal AAV-TNF led to decreased body weight. It increased conditioning to but not subsequent memory of an aversive context, suggesting impaired consolidation; it also increased anxiety. Amygdala AAV-TNF was without effect on body weight and aversive stimulus learning-memory, but reduced saccharin consumption and increased anxiety. This study adds significantly to the evidence that both peripheral and brain region-specific increases in TNF lead to both sickness and depression- and anxiety disorder-relevant behavior and do so via different pathways. It thereby highlights the complexity in terms of indirect and direct pathways via

which increased TNF can act and which need to be taken into account when considering it as a therapeutic target.

Keywords

TNF; AAV-vector; sickness; depression; anxiety disorders; cytokine hypothesis; fear learning-memory; anxiety; reward

Highlights

- Blood TNF induces sickness, reduces reward drinking and increases fear memory
- AAV-TNF vector induces CNS region-specific increased TNF and microglia activation
- Hippocampal TNF induces sickness, impairs memory and increases anxiety
- Amygdala TNF reduces reward drinking and increases anxiety

1. Introduction

Major depressive disorder (hereafter depression) is a heterogeneous mental illness with symptoms including sadness, amotivation, helplessness, fatigue, weight change, sleep disturbance, impaired cognition, and suicidality (American Psychiatric Association, 2013; World Health Organization, 1992). Its prevalence is increasing and by 2020 depression is predicted to be the second major cause of disability worldwide (Murray and Lopez, 1996). It has a high co-morbidity with anxiety disorders (American Psychiatric Association, 2013). The etio-pathophysiology of depression remains poorly understood, however, and with current-generation therapies only one third of patients reach full remission (Warden et al., 2007). Recently, human studies have linked depression with inflammation, including the pro-inflammatory cytokine network (Dantzer et al., 2008; Maes, 1995; Miller et al., 2009; Smith, 1991). Depression exhibits high comorbidity with chronic inflammatory diseases including rheumatoid arthritis and multiple sclerosis, and is prevalent in individuals undergoing chronic cytokine therapy (Dantzer et al., 2008). The term sickness is often used to describe the weight loss, amotivation, fatigue and impaired cognition that occur in such patients (and indeed those with common pathogen infections), and the overlap with depression - at the symptom level at least - is clear. Tumor necrosis factor alpha (TNF-α, hereafter TNF), a pro-inflammatory cytokine, exhibits increased levels in blood (Dowlati et al., 2010) and brain (in its transmembrane form, (Dean et al., 2010)) in depression patients. Indeed, peripheral blockers of TNF action are under clinical investigation for treatment of depression: with the monoclonal TNF antibody infliximab, efficacy was demonstrated in a sub-group of patients with high baseline plasma CRP and TNF levels (Raison et al., 2013), and with the TNF blocker etanercept, depression and fatigue scores were reduced in psoriasis patients (Tyring et al., 2006).

Animal studies are essential to investigate for causality between increased TNF levels in the circulation or brain, and physical, behavioral and neurobiological changes relevant to sickness and depression. Whilst only a small number of such studies have been conducted to date, they have yielded supportive evidence for important TNF effects. In mice, acute TNF administration in the periphery led to reduced body weight and less social exploration (Bluthé et al., 1994) and reduced operant self-stimulation of lateral hypothalamus (van Heesch et al., 2013). Acute delivery of TNF into the lateral brain ventricles (intra-cerebroventricular, i.c.v.) also reduced social exploration (Bluthé et al., 1994; Palin et al., 2007, 2009). Furthermore, it reduced active responding in the forced swim test and tail suspension test, and decreased consumption of the gustatory reward, sucrose (Kaster et al., 2012). In the latter study, these TNF effects were prevented by i.c.v. co-administration of a TNF antibody (Kaster et al., 2012). Animal manipulations that model infection or chronic inflammatory disease and induce, as one of a constellation of immune responses, increased TNF levels, also lead to sickness and depression and anxiety disorder-relevant behavioral changes. For example, administering mice with peripheral lipopolysaccharide (LPS) to activate the innate immune system, led to increased plasma TNF (Biesmans et al., 2013; Dantzer et al., 2008) and brain *Tnf* mRNA (Lawson et al., 2013), and loss of body weight, reduced social exploration (Bluthé et al., 2000) and sucrose drinking (Frenois et al., 2007; Lawson et al., 2013). Also in mice, immune activation of the TNF receptor superfamily member CD40, important in the host-pathogen response and autoimmune disorders, induced increased levels of TNF in plasma and of *Tnf* mRNA, although not TNF protein, in brain, whilst reducing body weight and feeding, increasing sleep, reducing sucrose drinking and impairing learning; aspects of the immune response and the behavioral effects were prevented by peripheral TNF blocking with etanercept (Gast et al., 2013; Cathomas et al.,

2015). Murine cytomegalovirus induced increased levels of TNF and interleukins in blood and of their respective genes in brain, with TNF contributing to an observed hyper-anxiety (Silverman et al., 2007). Experimental autoimmune encephalomyelitis (EAE) in mice, a model of multiple sclerosis, led to reduced activity in the center of an open field and impaired learning-memory, concurrent with increased brain TNF levels and signaling; certain of these effects were blocked by i.c.v. etanercept (Acharjee et al., 2013; Haji et al., 2012; Habbas et al, 2015). Chronic psychosocial stress in mice has also been demonstrated to increase TNF and interleukin levels and to lead to depression- and anxiety disorder-relevant behavior (Azzinnari et al., 2014; Fuertig et al., 2016; Kinsey et al., 2008).

Therefore, there is rodent evidence that acute and specific increases in TNF in either the periphery or brain lead to weight loss and specific changes in behavior of relevance to depression and anxiety disorder, with the two routes of TNF administration leading to similar effects. Furthermore, there is rodent evidence that more prolonged increases in TNF in periphery and brain, induced as part of a constellation of immune changes by LPS or CD40-activation, lead to weight loss, other markers of sickness, and depression- and anxiety disorder-relevant behavior. Against this background, the aims of the current mouse study were to investigate and directly compare the effects of (1) continuous/daily-repeated peripheral TNF administration versus (2) continuous brain region-specific viral vector TNF expression on: inflammation in periphery and brain, body weight, emotional behavior, and memory. The behavioral tests utilized were the two-bottle saccharin versus water test for assessment of interest in gustatory-reward; general context or specific auditory-stimulus conditioning to footshock for assessment of fear reactivity, and next-day test of fear expression for assessment of memory; two-way approach-avoidance conflict test for assessment of anxiety. In the research domain criteria (RDoC) framework for mental health

research, reward interest is a dimension in the domain Positive valence systems, fear and anxiety are in the domain Negative valence systems, and memory is in the domain Cognitive systems (Cuthbert and Insel, 2013). The observed constellations of immune, physical and behavioral effects induced by TNF were compartment- and brain region-specific. This study adds significantly to the evidence that a sustained increase in either peripheral or central TNF exerts effects on sickness and emotional and cognitive behaviors, with the latter effects being brain region-dependent. It highlights the complexity in terms of indirect and direct pathways via which increased TNF can act and which need to be taken into account when considering it as a therapeutic target for the treatment of sickness and affective pathologies.

2. Materials and Methods

2.1. Animals and maintenance

Male C57BL/6J mice (Janvier Labs, Le Genest-Saint-Isle, France) were delivered in littermate pairs and accustomed to the new environment for two weeks. Mice were aged 10 to 12 weeks and weighed 25.0 to 30.0 g at study onset. They were maintained on a reversed 12:12 h light-dark cycle (lights off 07:00-19:00) in an individually-ventilated caging system (IVC) at 20-22 °C and 50-60 % humidity. Cages were type 2L and contained woodchips, a sleep igloo and tissue bedding. Complete-pellet diet (Provimi, Kliba Ltd, Kaiseraugst, Switzerland) and water were available continuously. In the week prior to the onset of an experiment, mice were handled on three days. The study was conducted under a permit (170/2012) for animal experimentation issued by the Veterinary Office, Zurich, Switzerland. All efforts were made to minimize the number of mice used and unnecessary stress.

2.2. Experiments with repeated i.p. TNF injection

Daily repeated peripheral injection of recombinant murine TNF (ImmunoTools, Friesoythe, Germany) was performed intra-peritoneally (i.p.) in non-anaesthetized mice. TNF was reconstituted in dH_2O vehicle and, based on previous studies (Bluthé et al., 1994; van Heesch et al., 2013), injected daily at a concentration of 20 or 40 ng/g body weight (in a 25 g mouse, equivalent to 0.5 and 1.0 µg/day, respectively) on consecutive days. Mice were maintained in littermate pairs with both mice per pair assigned to the same dose group.

Naive mice (n=22) were studied to investigate effects on TNF plasma and brain levels, and on body weight as a sickness measure. Mice were allocated randomly to TNF at 40 ng/g (IP-TNF40, n=11) or vehicle (IP-VEH, n=11). Injections were given at 16:00 on 10 consecutive days. Body weight was taken daily at 16:00. On day 9, from a subset of IP-TNF40 (n=5) and IP-VEH (n=5) mice, tail-vein blood samples were collected at 1, 3 and 6 h post-injection, for plasma TNF determination. On day 10, 1 h after the last injection, all IP-TNF40 and IP-VEH mice were decapitated. In one subset (IP-TNF40 n=4, IP-VEH n=4), trunk blood was collected for TNF, IL-6 and IL-10 determination and brains were extracted and frozen on dry ice for TNF determination. In the other subset (IP-TNF40 n=6, IP-VEH n=6) the spleen was extracted, cleaned and weighed.

To assess whether repeated i.p. TNF administration results in passage of TNF into the brain, naive mice were injected i.p. with human TNF. Human and murine TNF protein structures are similar (Brouckaert et al., 1986; Kuprash et al., 1999) and would be expected to exhibit similar peripheral-brain passage (Banks et al., 1995), which in turn is dependent on the inflammatory environment (Erickson and Banks, 2011). Naive mice were injected daily for 5 days at 16:00 with human TNF (ImmunoTools) at a dose of 40ng/g IP-hTNF (low hTNF, n=3) or IP-VEH (VEH, n=3), or once at a dose of 400ng/g IP-hTNF (high hTNF, n=3). At 1 h after the final injection, mice were decapitated and trunk blood and brains were collected

and processed for TNF determination.

For fear conditioning (see section 2.5.1), naive mice (n=32) were allocated randomly to three groups: TNF at 20ng/g (IP-TNF20, n=10), TNF at 40 ng/g (IP-TNF40, n=10), and vehicle (IP-VEH, n=12). Injections were given at 16:00 on 4 consecutive days. On days 4-5 at 08:30-12.30, mice underwent fear conditioning and expression testing, respectively. In naive mice (n=24) studied in the two-bottle saccharin test (see section 2.5.2), where day 1 was the first day of TNF injection, baseline water consumption was measured on days -7 to -5, and baseline saccharin consumption and preference were measured on days -4 to 0. Counter-balancing on baseline saccharin preference scores, mice were allocated to IP-TNF20 (n=8), IP-TNF40 (n=8), IP-VEH (n=8). TNF injections were given on days 1 to 6 at 16:00, and water and saccharin consumption were measured on days 4 to 9. As well as providing data for baseline consumption, pre-exposure of mice to saccharin prior to i.p. TNF injection also prevented taste-conditioned aversion related to any TNF-induced sickness (Cathomas et al., 2015).

2.3. Experiments with osmotic micropump s.c. TNF delivery

With the aim of attaining continuous peripheral delivery of murine TNF, osmotic micropumps delivering 0.5 µl/h for 7 days (model 1007D, Alzet, Cupertino, USA) were implanted subcutaneously. In vitro experiments established appropriate conditions for TNF delivery: bovine serum albumin (0.5% w/v) was added to PBS 1x as vehicle. A pump containing 20 µg/ml TNF was placed in a 2 ml protein LoBind tube filled with 0.9% NaCl + 0.5% BSA to mimic the subcutaneous space; constant TNF delivery at 40% of expected concentration was observed with loss attributed to binding and TNF denaturation. For in vivo experiments, osmotic pumps were filled according to manufacturer's instructions with 100 µL 40 µg/ml murine TNF in PBS 1x + 1% BSA as vehicle, or vehicle only. For implantation,

mice were anesthetized with a triple anesthesia of fentanyl, midazolam and medetomidine (2.4 µl/g) i.p., a small incision was made between the scapulae and the pump was positioned caudally to the incision, which was sutured. Mice were injected i.p. with an anesthetic-antagonist of flumazenil and atipamezole (7.2 µl/g). Mice were singly housed thereafter. Given that the pump delivered 0.5 µl/h and 12 µl/day, expected delivery was 480 ng TNF/day (e.g. ca. 20 ng/g body weight in a 25 g mouse) and therefore comparable to the daily doses in the repeated i.p. TNF experiments.

Naive mice were implanted on day 0 with an osmotic pump containing 40 µg/ml TNF (OP-TNF40, n=6) or vehicle (OP-VEH, n=9). All mice were weighed daily at 16:00. On days 1, 3 and 6, a tail-vein blood sample was collected for plasma TNF determination, and on day 7 mice were decapitated and trunk blood was collected for determination of plasma TNF, IL-6 and IL-10. Spleens were removed, cleaned and weighed. To control whether pumps had delivered the expected volume, the residual volume was withdrawn and measured post-experiment.

Naive mice were assessed in the two-bottle saccharin test. Where day 0 was the day of pump implantation, mice were habituated to drinking bottles on day -14, on days -12 to -10 baseline water consumption was measured, and baseline saccharin consumption and preference on days -7 to -3. Mice were allocated to two groups, OP-TNF40 and OP-VEH (n=6/group). Water and saccharin consumption were measured on days 1 to 5. Body weight was measured daily.

2.4. Experiments with brain region-specific AAV-vector TNF expression

2.4.1 Construction and production of viral vectors

Construction of self-complementary adenovirus-associated virus vector plasmids

Self-complementary (sc) adenovirus-associated virus (AAV) vector plasmids (p) were constructed as described (McCarty et al., 2003; Wang et al., 2003). Briefly, the terminal resolution site (trs) and the D-sequence (the packaging signal) were deleted by *Bal* I restriction within the AAV serotype 2 (AAV-2) 5' inverted terminal repeat (5'-ITR) of psub-2-CBA-WPRE (Paterna et al., 2000, 2004), resulting in pscAAV-2-Δ3'-ITR. Subsequently, the multiple cloning site (MCS) of pBluescript II SK (+) (Stratagene) together with the AAV-2 3'-ITR and simian virus 40 late polyadenylation signal [SV40p(A)] containing fragment of psub-2-CMV-WPRE (Paterna et al., 2000) were inserted into pscAAV-2-Δ3'-ITR, resulting in pscAAV-2-MCS-SV40p(A). The human cytomegalovirus (hCMV) promoter/immediate-early enhancer (IE) of pEGFP-N1 (Clontech) and the chimeric intron (chI) of pSI (Promega) were inserted into pscAAV-2-MCS-SV40p(A), resulting in pscAAV-2-hCMV-chI-SV40p(A). The EGFP open reading frame (ORF) of pEGFP-N1 was PCR-amplified using primers containing direct repeated, reverse oriented loxP sites and inserted into pscAAV-2-hCMV-chI-SV40p(A), resulting in pscAAV-2-hCMV-chI-floxedEGFP-SV40p(A). The murine TNF-alpha ORF was isolated from total murine RNA by reverse transcription PCR and inserted in place of the EGFP ORF into pscAAV-2-hCMV-chI-floxedEGFP-SV40p(A), resulting in pscAAV-2-hCMV-chI-floxed mTNFalpha-SV40p(A). In order to generate a control AAV vector devoid of an ORF, the mTNF-alpha ORF and the 3'-loxP site within pscAAV-2-hCMV-chI-floxedmTNFalpha-SV40p(A) were removed, resulting in pscAAV-2-hCMV-chI-loxP-SV40p(A). The integrity of all constructs was confirmed by Sanger DNA sequencing and restriction endonuclease analysis. The 3'-loxP site was not required for the current experiments.

Production and purification of scAAV-8/2 vectors (capsid proteins of AAV-8, ITRs of AAV-2)

The scAAV vectors, scAAV-8/2-hCMV-chI-floxedEGFP-SV40p(A), scAAV-8/2-hCMV-chI-floxed mTNFalpha-SV40p(A) and scAAV-8/2-hCMV-chI-loxP-SV40p(A), were produced and purified as described (Paterna et al., 2004; Zolotukhin et al., 1999). Briefly, human embryonic kidney (HEK) 293 cells (Graham et al., 1977) expressing the simian virus (SV) large T-antigen (293T) (DuBridge et al., 1987) were transfected by transient calcium phosphate- or polyethylenimine (PEI)-mediated co-transfection of AAV vector (see above), AAV helper (p5E18-VD2/8 (Gao et al., 2002)) and adenovirus (AV) helper (pBS-E2A-VA-E4 (Paterna et al., 2000)) plasmids. At 72 hours post-transfection, HEK 293T cells were collected, supernatant discarded, and cells lysed by 4 freeze-thaw cycles (liquid nitrogen, -37 °C water bath) or by Bertin's Minilys Homogenizer (VWR) in combination with 7 ml soft tissue homogenizing CK14 tubes (VWR). The crude cell lysate was treated with Benzonase® endonuclease and cleared by centrifugation. The cleared lysate was subjected to discontinuous density iodixanol (OptiPrep™, Axis-Shield) gradient (isopycnic) ultracentrifugation. Subsequently, the iodixanol was removed from the fraction containing AAV vector by diafiltration (ultrafiltration) using Vivaspin 20 ultrafiltration devices (100'000 MWCO, PES membrane, Sartorius) and 1x PBS supplemented with 1 mM $MgCl_2$ and 2.5 mM KCl. The AAV vectors were aliquoted and stored at -80 °C.

Quantification of scAAV-8/2 vectors

Encapsidated viral vector genomes (vg) were quantified using the Qubit™ 3.0 fluorometer (Life Technologies) in combination with the Qubit™ dsDNA HS Assay Kit (Life Technologies). Briefly, 10 µl of untreated and 10 µl of heat-denatured (5 minutes at 95 °C) AAV vector were quantified according to the manufacturer's instructions. Intraviral (encapsidated) vg/ml was

calculated by subtracting the extraviral (non-encapsidated; untreated sample) from the total intra- and extra-viral (encapsidated and non-encapsidated; heat-denatured sample).

AAV vector injection procedure and dose-finding

Stereotaxic bilateral injection of AAV vector was performed using coordinates informed by a mouse brain atlas (Paxinos and Franklin, 2008). The AAV vector was adjusted to its working dilution in PBS-MK. Mice were anesthetized by i.p. injection of a mix of fentanyl, midazolam and medetomidine (2.4 µl/g) and placed in a stereotaxic frame. After a skin incision and exposure of the skull, the bregma was used to zero all coordinates using a binocular microscope and a hole was drilled at the desired coordinates. Using a microinjector (SP210iwZ, WPI, Berlin, Germany) fitted with a needle with outer diameter of 210 µm (Nanofil, 33G, WPI), mice were injected bilaterally with 1 µl per hemisphere at a rate of 200 nl/min. After injection, the needle remained in place for 5 min to limit reflux along the injection tract, followed by slow retraction. The skin over the skull was closed with surgical clips, the incision site disinfected, and mice were injected i.p. with an anesthetic-antagonist of flumazenil and atipamezole (7.2 µl/g).

To determine the working titer for the AAV-TNF vector, a range of titers was microinjected into the medial hippocampus: 1.1e10 vg/ml, 1.1e11 vg/ml or 1.1e12 vg/ml were used, injecting 1 µl unilaterally. After 14 days, the TNF concentration in tissue lysates from medial hippocampus was measured and normalized to protein titer (see Section 2.7. for details). In a second pilot experiment, the AAV-EGFP vector was injected i.c.v. at a titer of 5e12 vg/ml to identify those regions that take up AAV-vector through this route; after 14 days of expression, brains were processed for fluorescence microscopy. EGFP expression was visualized in the following regions: fimbria hippocampi, surrounding the lateral and third ventricles i.e. paraventricular thalamic nucleus, habenula, stria terminalis, dentate gyrus,

para-, supra and peri-ventricular hypothalamic nuclei (hereafter termed periventricular regions), also hypothalamus, hippocampus, aqueduct and pontine nuclei (see Results, Figure S2). Pilot behavioral experiments conducted with mice injected with the AAV-EGFP vector in lateral ventricle, hippocampus or amygdala identified mild but significant behavioral effects relative to mice injected with the control vector (AAV-CON vector, no ORF) or with PBS (Baens et al., 2006). EGFP acts on several signaling pathways including the NF-κβ pathway of cytokine gene expression, and is therefore not a suitable control protein in certain experimental designs as reported previously (Baens et al., 2006). Therefore, AAV-CON was used as the control vector throughout the study.

2.4.2. AAV-TNF target regions and experiments

The target regions for the study of AAV vector-mediated increased TNF expression were lateral ventricle, hippocampus and amygdala. In all experiments, 14 days were allowed between injection and onset of behavioral testing, for surgical recovery and vector expression. Mice were maintained in littermate pairs with both mice per pair assigned to the same vector group.

Lateral Ventricle (i.c.v.)

Naive mice (n=26) were used to investigate the effects of increased TNF levels following bilateral i.c.v. injection of AAV vector. The coordinates were anteroposterior (AP) 0.2 relative to bregma, mediolateral (ML) 0.5/-0.5, dorsoventral (DV) -0.28. Mice were injected with either AAV-TNF vector at 1.1e11 vg/ml (n=14) or AAV-CON at 1.1e10 vg/ml (n=6, n=12 with hippocampal AAV-CON mice, see below). Injections were performed on day 0. On days 14-15, mice were tested in contextual fear conditioning, on day 16 in the two-way avoid-escape test and on days 18-22 a subset of mice was tested in the two-bottle saccharin test, comprising water consumption on days 18-19 and saccharin consumption and preference on

days 20-22. After the behavioral testing on day 22, mice were decapitated, trunk blood was collected and brains were fresh frozen for TNF determination. Mice injected with AAV-CON in hippocampus (n=6) exhibited equivalent behavior and region-specific TNF levels to these i.c.v. AAV-CON mice, and therefore the behavioral data from the two groups were pooled to give n=12 per region. In naive mice, AAV-TNF (n=6) or AAV-CON (n=6) were injected and after 14 days trunk blood was collected for determination of plasma levels of TNF, IL-6 and IL-10 at this time point.

Hippocampus

Naive mice (n=28) were used to investigate effects of bilateral increased TNF levels in medial hippocampus. The coordinates were AP -2.80, ML 2.5/-2.5, DV -1.8. Mice were injected with either AAV-TNF at 1.1e10 vg/ml (n=16) or AAV-CON at 1.1e10 vg/ml (n=6, n=12 with i.c.v. AAV-CON mice). On days 14-15 mice were tested in contextual fear conditioning, on day 16 in the two-way avoid-escape test, and on days 18-22 a subset of mice was tested in the two-bottle saccharin test, comprising water consumption on days 18-19 and saccharin consumption and preference on days 20-22. After the behavioral testing on day 22, mice were decapitated, trunk blood was collected and brains were fresh frozen for TNF determination.

Amygdala

Naive mice (n=24) were used to investigate effects of bilateral increased TNF levels in amygdala. The coordinates were AP -1.06, ML 2.8/-2.8, DV -4.95. Mice were injected with either AAV-TNF at 1.1e10 vg/ml (n=12) or AAV-CON at 1.1e10 vg/ml (n=12). On days 14-15 mice were tested in auditory-stimulus fear conditioning, on day 16 in the two-way escape test, and on days 18-22 in the two-bottle saccharin test comprising water consumption on days 18-19 and saccharin consumption and preference on days 20-22. After the behavioral

testing on day 22, mice were decapitated, trunk blood was collected and brains were fresh frozen for TNF determination. A separate cohort of naive mice (n=9) was studied for the effects of increased amygdala TNF expression on plasma levels of TNF, IL-6 and IL-10; mice were injected with either AAV-TNF (n=6) or AVV-CON (n=3) at the titers used for behavioral testing. A further cohort was studied for the effects of increased amygdala TNF on microglial activation using Iba1 immunohistochemistry (see section 2.8): mice were injected with AAV-TNF (n=5), AAV-CON (n=3) or VEH only (n=3).

2.5. Behavioral testing

2.5.1. Pavlovian fear conditioning-memory and two-way avoid-/escape anxiety

Two strategies were used for testing behavior to aversive stimuli: (1) CS-US fear conditioning test followed by two-way US escape test; (2) context-US fear conditioning test followed by two-way CS-US avoid-escape test. Mice in the i.p. TNF and amygdala TNF experiments were tested using strategy 1, with the emphasis on CS fear learning-memory, which are amygdala dependent (Johansen et al., 2011). Mice in the i.c.v. and hippocampus TNF experiments were tested using strategy 2, with the emphasis on context fear learning-memory, which are hippocampus dependent (Maren et al., 2013; Orsini and Maren, 2012). Testing was conducted between 08:00-14:00, using a Multi Conditioning system (TSE Systems GmbH, Bad-Homburg, Germany), details of which are given in (Azzinnari et al., 2014; Cathomas et al., 2015a; Pryce et al., 2012). Briefly, a Plexiglas arena was placed on an electrified grid floor, and a frame containing an infrared movement detection system in X, Y, Z planes surrounded the arena/grid. Four such units were each contained in an attenuating chamber with a ventilation fan, house lights (8 lux) and a loud speaker emitting low-level white noise. Prior to each session the arenas and the grids were wiped with 70% ethanol and the waste

tray was rinsed and dried.

CS-US fear conditioning-memory and two-way US escape anxiety test

The conditioning session comprised 6 trials of 20 s x 85 dB x 5 kHz tone conditioned stimulus (CS), the final 2 s of which overlapped with 0.2 mA inescapable footshock unconditioned stimulus (US). The interval between successive CS-US trials (inter-trial interval, ITI) was 120 s. Measures were % time freezing (no detection of any movement for a minimum of 2 s) during CS and ITI. In addition, distance moved during the US (US reactivity) was measured as a non-specific marker of pain sensitivity, fear and arousal. On the following day, consecutive tests of fear expression to the context and CS were conducted. For contextual fear expression, the mouse was returned to the same arena without CS or US for 21 x 60 s intervals and % time spent freezing was measured. Directly afterwards, in the same context, the CS fear expression test was conducted, comprising 12 x 30 s CS separated by 90 s ITI, and % time freezing during CS was measured. On the next day, the two-way US escape test was conducted: the same arena was divided into two identical compartments by a black divider that contained a gate so that mice could transfer readily between compartments. The test comprised 30 trials of 0.2 mA escapable US with a maximum duration of 5 s and with ITI of 50 s. One transfer during the US terminated it and comprised an escape response, and absence of US transfer was an escape failure. Measures of interest were escapes, escape failures, % time freezing to ITI, ITI transfers and US reactivity.

Context-US fear conditioning-memory and two-way CS-US avoid-escape anxiety test

The conditioning session consisted of 6 inescapable footshocks of 2 s x 0.20 mA delivered at ITI of 120 s; % time freezing during ITI and US reactivity were measured. On the following day, the context fear expression test comprised 11 x 60 s intervals in the same arena; % time

freezing per 60 s interval was measured. On the next day the two-way CS-US avoid-escape test was conducted. The session comprised 30 CS trials of 85 dB x 5 kHz tone with a maximum duration of 10 s, the offset of which was coincident with the onset of a 0.2 mA escapable US with a maximum duration of 5 s, and ITI of 50s. One transfer between compartments during the CS immediately terminated the CS and resulted in avoidance of the US (avoid response); one transfer after the CS and during the US immediately terminated the US (escape response); absence of both avoid and escape responses resulted in 10 s CS and 5 s US (avoid-escape failure). Measures of interest were avoids, escapes, avoid-escape failures, % time freezing to CS, ITI transfers and US reactivity.

2.5.2. Two-bottle saccharin versus water test

A divider was used to form two compartments in the home-cage, and mice were habituated to being in separate compartments and to drinking from 15 ml polypropylene centrifuge tubes (Falcon, BD, Franklin Lakes, USA) from which the tip had been removed (Cathomas et al., 2015b). Each mouse was presented with two such tubes placed adjacently and fixed to the cage lid. These conditions were set up from 08:00 h to 16:00 h on each day of training and testing. Tubes were either filled with water or 0.05% saccharin in water (w/v) (saccharin sodium salt hydrate, Sigma-Aldrich, Buchs, Switzerland), with the left-right positioning of the two tubes alternated across days. Amount drunk per tube was estimated as tube weight (g) at 08:00 h – tube weight (g) at 16:00 h, and % saccharin preference was calculated as: (saccharin solution consumed/(saccharin solution + water consumed)) x 100. The two-bottle test allows for assessment of effects on the absolute level of reward consumption as well as the preference relative to water; if baseline reward consumption is high and little water is consumed, as in the present study, then absolute consumption provides the more sensitive measure.

2.6. Blood, brain and spleen collection and preparation

Tail-vein blood samples (100 µl) were collected into chilled EDTA capillary tubes (Microvette CB 300 K2E, Sarstedt, Nürnbrecht, Germany). Following rapid decapitation, trunk blood (400-500 µl) was collected into chilled EDTA-coated tubes (Microvette 500 K3E, Sarstedt). Bloods were centrifuged at 3000 rpm and 4 °C for 15 min, and plasma aliquots were transferred to cryotubes (Protein LoBind, Eppendorf, Basel, Switzerland) and stored at -80 °C. Following decapitation, the brain was removed from the skull, rinsed with ice-cold saline, frozen on powdered dry ice and stored at -80 °C. The spleen was removed, cleaned of fat and connective tissue, and weighed.

2.7. Plasma and brain-tissue cytokine immunoassay

In plasma samples, the concentrations of murine TNF, IL-6 and IL-10 were determined using a multiplexed particle-based flow cytometric cytokine assay according to a published procedure (Marques-Vidal et al., 2011; Cathomas et al., 2015). For TNF, the range of the standard curve was 0.1-30 pg/ml, and the lower limit of quantification (LLOQ) was 0.3 pg/ml. For TNF determination in specific brain regions, fresh frozen brains were cut coronally into sections of 1 mm in a mouse brain matrix at -18 °C and processed according to a previously published protocol (Azzinnari et al., 2014). Briefly, from the corresponding sections, brain regions of interest were microdissected from each hemisphere using a tissue biopsy micropunch (∅ = 1 mm): amygdala (1 biopsy/hemisphere) at bregma -0.8 to -1.8 ± 0.3 mm; medial hippocampus (2 biopsies/hemisphere) at bregma -2.3 to -3.4 ± 0.3 mm; and periventricular regions (surrounding lateral and third ventricle, i.e. paraventricular thalamic nucleus, habenula, stria terminalis, dentate gyrus, para-, supra and peri-ventricular hypothalamic nuclei, 5 biopsies in total) at bregma -0.2 to -1.1 ± 0.2 mm. Tissue biopsies per region/mouse were pooled and stored in a 1.5 ml ProteinLoBind polypropylene tube

(Eppendorf, model 022431081) at -80 °C. Tissue biopsies (0.5-4.0 mg) were lysed using MSD complete tris lysis buffer (MesoScaleDiscovery, Maryland, USA), a conventional tris lysis buffer containing EDTA and EGTA with the addition of protease inhibitor solution (Pierce 78439, Thermo Fisher Scientific, Massachusetts, USA) and phosphatase inhibitor solution I and II (P-0044 and P-5726, Sigma-Aldrich), each at 0.01%. 100 µl of ice-cold MSD complete tris lysis buffer was added to each tube containing tissue biopsies and homogenized using ultrasonication of 2x 5 sec. Lysed tissue was left on ice for 20 min and then centrifuged at 14'000 rpm for 10 min. The supernatant was pipetted into separate, pre-chilled Eppendorf tubes and used for protein and cytokine determination. Determination of murine TNF in brain-tissue lysates was performed using the same assay as for plasma samples, following validation: accuracy, calculated as recovery of TNF added to lysate containing a low level of TNF, was 95 %; LLOQ was 0.3 pg/ml lysate; intra-assay precision was 5.2 % (coefficient of variation, N=20 from a lysate sample pool); serial dilution of lysate pools yielded TNF values that ran in parallel to the standard curve. For human TNF following i.p. injection, an electrochemiluminescence assay was used (VPlex small spot kit for human TNF, MesoScaleDiscovery) according to the manufacturer's protocol. The LLOQ was 1 pg/ml. The assay was without cross-reactivity with murine TNF. The total protein content of lysates (2 µl/well in duplicate) was determined using Coomassie plus protein assay reagent with a bovine serum albumin standard curve (in triplicate) and the manufacturer's microtitre plate protocol (Thermo Fisher Scientific).

2.8. Microglial Iba-1 immunohistochemistry following amygdala AAV-TNF

Mice from the amygdala AAV-TNF experiment (AAV-TNF (N=5), AAV-CON (N=3) and Vehicle (N=3)) were deeply anesthetized (pentobarbital 5 mg in 0.1 ml i.p.) and transcardially perfused with 1x PBS (40 ml) followed by 4% paraformaldehyde (PFA, 120 ml). Brains were

removed and postfixed in PFA at 4 °C for 24 h, then cryoprotected in 30 % sucrose in PBS for 2 days, and frozen. Using a sliding microtome, brains were sectioned coronally at 20 µm and cryosections were collected in antifreeze solution and stored at -20 °C. Sections including the amygdala, 8-10/mouse, were assessed for microglial activation in terms of ionized calcium-binding adapter molecule 1 (Iba-1) signal, as a biomarker of TNF expression-function. Endogenous peroxidase was inhibited using pre-incubation with 3 % H_2O_2 for 15 min, followed by 2x 10 min washing in TBS+ (TBS2x, 0.05 % Triton). Non-specific binding sites were blocked with blocking solution (2 % goat serum, 0.3 % Triton in TBS2x) for 45 min. Primary antibody rabbit anti-mouse Iba-1 (Wako, Neuss, Germany) was added into the blocking solution at a concentration of 1:1000 and incubated at 4 °C overnight. Sections were washed 2x 10 min in TBS+, and the secondary goat anti-rabbit antibody (1:250, Millipore) was added and incubated for 2 h. Following two further washes in TBS2x, sections were incubated for 30 min at RT with 1 % avidin-biotin-peroxidase complex (Vectastain Elite Kit, Vector Laboratories, Reactolab, Servion, Switzerland) diluted in TBS1x. Following 2x 10 min washing, DAB-Nickel staining was performed with a kit (Metal Enhanced DAB Substrate Kit, Thermo Scientific) according to the manufacturer's protocol. The reaction was stopped after 90 sec by washing 2x 10 min in ice-cold TBS1x. Sections were then mounted on superfrost plus slides (Thermo Scientific), air-dried, dehydrated and cover-slipped. Sections were analyzed with bright-field microscopy (Axio Observer.Z1, Zeiss, Feldbach, Switzerland). Images of the amygdala were acquired at 20x magnification, with an exposure time of 13.4 ms. The sampling frame was placed manually according to anatomical landmarks to cover an area comprising the majority of the basolateral and central nuclei of the amygdala and the corresponding extents of the internal and external capsules. Quantification of the area of positive staining in the sampled images was carried out using ImageJ (Rasband, W.S., ImageJ,

U. S. National Institutes of Health, Bethesda, Maryland, USA, 1997-2014), including the settings, *thresholding*, *background subtraction*, and *particle analysis by size and circularity*. The mean value for the left and right amygdala of each section was calculated. From the 8-10 sections/mouse, the three yielding the highest positive-labeled mean areas were selected, and their mean value was calculated and used for analysis.

2.9. Statistical analysis

Statistical analysis of TNF effects on physical, physiological and behavioral measures was conducted using SPSS (Version 21, SPSS Inc., Chicago IL, USA). Linear mixed model was used, with a fixed effect of group (TNF, VEH), and also of trial in readout tests with multiple trials, and a random effect of mouse in the case of multiple trials. In the case of significant main or interaction effects, post hoc testing was conducted using the least significant difference (LSD) test. Statistical significance was set at $p<0.05$. The estimate of variance used is one standard deviation (SD).

3. Results

3.1. Repeated i.p. TNF injection

3.1.1. Physiological effects and peripheral-brain passage

In mice i.p. injected with TNF 40ng/g (IP-TNF40) or vehicle (IP-VEH) on days 1-10, tail blood was collected on day 9 at 1 h, 3 h and 6 h after the injection for plasma TNF determination. There was a significant group x time interaction ($F(2,14)=125.54$, $p<0.0005$; Fig. 1A): post hoc testing confirmed that IP-TNF40 mice had markedly elevated plasma TNF relative to IP-VEH mice at 1 h ($p<0.0005$) and also 3 h ($p<0.002$) post-injection, and that plasma TNF had returned to baseline levels at 6 h post-injection. Plasma levels of IL-6 and IL-10 were also

determined in samples collected on day 10; levels of both cytokines were below LLOQ in IP-VEH mice, whereas IL-6 levels of 4-10 pg/ml were measured in IP-TNF40 mice and IL-10 was detectable in two of these mice (Fig. 1B). For absolute body weight at days 1-8 there was a group x day interaction ($p<0.03$) but no day-specific group post hoc effect. When body weight at days 1-8 was analyzed relative to day 0 baseline, there was a group main effect ($F(1,20)=11.01$, $p<0.003$) with IP-TNF40 mice exhibiting lower relative body weight than IP-VEH mice (Fig. 1C). On day 10, 1 h after the last injection, there was a borderline increase in absolute spleen weight in IP-TNF40 mice ($p=0.07$) and an increase in spleen weight (mg) relative to body weight (g) in IP-TNF40 (3.26 ± 0.65) relative to IP-VEH mice (2.56 ± 0.14) ($t(10)=2.59$, $p<0.03$). On day 10, 1 h after the last injection, brain TNF levels were analyzed (Fig. 1D). In periventricular regions there was a borderline increase in TNF level in IP-TNF40 versus IP-VEH mice ($t(8)=2.25$, $p=0.055$). There was no group effect in medial hippocampus or amygdala (Fig. 1D). Comparing TNF levels across brain regions, there was a main effect of region ($F(2,16)=26.71$, $p<0.0005$), with higher levels in amygdala than in periventricular regions and hippocampus ($p<0.0005$) (Fig. 1D).

In a separate cohort, mice were injected i.p. on 5 days with 40 ng/g human TNF, equivalent to the murine TNF dose used, or IP-VEH. At 1 h after the last injection, brains were collected and region-specific human TNF was measured. An additional group of mice was injected once with 400 ng/g human TNF (high IP-hTNF) and decapitated 1 h after injection. As expected, brain hTNF levels were below assay LLOQ in IP-VEH mice (Fig. 1E). In periventricular regions 2 of 3 IP-hTNF40 mice exhibited detectable human TNF. In the amygdala and medial hippocampus human TNF was at LLOQ. Mice that received high IP-hTNF exhibited high human TNF in each brain region and particularly in periventricular regions (Fig. 1E).

Figure 1. Effects of 10 days of daily i.p. injection of murine TNF on physiologic markers

3.1.2. CS fear conditioning and memory

A naive cohort of mice was injected daily i.p. with TNF 20 ng/g (IP-TNF20), TNF 40 ng/g (IP-TNF40) or vehicle (IP-VEH) on days 1-4. On day 4, arena habituation and CS-US fear conditioning were conducted, and on day 5 tests of fear expression to context and then CS were conducted. On day 4, during the 5-min arena habituation session, there was a group effect on locomotor activity ($F(2,35)=3.62$, $p<0.04$): IP-TNF20 mice (49182±6504 a.u.) were more active than IP-VEH mice (39427±6253 a.u.), with IP-TNF40 mice (38224±15093 a.u.) exhibiting activity similar to IP-VEH mice. There was no group effect on baseline % time freezing ($p=0.38$) or on exploratory behavior as assessed by rearing ($p=0.08$) (data not shown). In CS-US fear-conditioning, fear learning was indicated by the main effect of CS-US trial on % time freezing during CS ($F(2,58)=39.80$, $p<0.0005$; Fig. 2A). There was no effect of TNF group ($p\geq0.54$). Percent time freezing during ITI also increased continuously ($F(2,58)=125.03$, $p<0.0005$) and again there was no effect of TNF group (data not shown). US reactivity did not differ between groups: IP-VEH (698±159 a.u.), IP-TNF 20 (711±105 a.u.), IP-TNF40 (670±173 a.u.) (main effect: $p=0.63$). On day 5, in the test of expression of contextual fear memory, there was a main effect of interval ($F(6,174)=8.84$, $p<0.0005$), with highest % time freezing (19-35 %) occurring in min 1-3 and subsequently declining. There was no effect of TNF group on % time freezing to context ($p\geq0.16$) (data not shown). In the test of expression of CS fear memory, there was a main effect of CS trial ($F(5,145)=7.58$, $p<0.0005$; Fig. 2B) with highest % time CS freezing occurring in CS trials 1-2. There was also a main effect of group ($F(2,29)=3.95$, $p<0.03$; Fig. 2B), with IP-TNF40 mice exhibiting increased % time freezing during CS relative to IP-VEH ($p<0.03$) and IP-TNF20 ($p<0.02$) mice.

Figure 2. Effects of daily i.p. injection on CS fear conditioning

3.1.3. Two-bottle saccharin test

A naive cohort of mice was tested in the two-bottle saccharin versus water test. On days -7 to -5, mice were given two water bottles to measure baseline water consumption, and on days -5 to -1 one saccharin and one water bottle to measure baseline saccharin consumption/preference and for saccharin pre-exposure to prevent taste-conditioned aversion. On day -1, average saccharin consumption was 5.59±0.17 g (n=24; see Fig. 3A) and average saccharin preference was 95±0.4 % (n=24). On days 1 to 6, mice received daily i.p. injections, and on days 4 to 9 mice were given one saccharin and one water bottle to assess TNF effects. There was no group effect on water consumption at days 4-9: IP-VEH (0.42±0.16 g), IP-TNF20 (0.38±0.17 g), IP-TNF40 (0.36±0.15 g) (group main effect: $p=0.42$). For saccharin consumption there was a group x day (4-9) interaction ($F(12,126)=2.38$, $p<0.008$; Fig. 3A): on days 4 and 5, IP-TNF40 mice consumed less saccharin than IP-VEH mice ($p<0.05$). A posteriori group-specific analysis based on day -1 (baseline) and days 4 to 9 identified no effect of day on saccharin consumption in IP-VEH mice ($p=0.20$; Fig. 3B). For IP-TNF20 mice there was a day effect ($F(6,42)=3.95$, $p<0.003$; Fig. 3C), with less saccharin drinking on day 6 versus baseline ($p<0.009$). For IP-TNF40 mice there was a day effect ($F(6,42)=14.62$, $p<0.0005$; Fig 3D), with less saccharin drinking on days 4-7 ($p<0.0005$) compared to baseline. For saccharin preference there was no group or day effect (e.g. group main effect: $p=0.90$, data not shown). These findings were not confounded by i.p. TNF effects on body weight, as demonstrated by re-analyzing the data as saccharin consumed in g per g body weight. There was a group x day effect on body weight ($p<0.0005$), reflecting lower body weight in IP-TNF20 and IP-TNF40 mice compared with IP-VEH mice, at days 4-5. For saccharin g/g body

weight, there was a group x day (4-9) interaction ($F(12,126)=2.00$, $p<0.003$): on days 4 and 5, IP-TNF40 mice consumed less saccharin/g body weight than IP-VEH mice ($p<0.05$) (Fig. S1A). A posteriori group-specific analysis based on day -1 (baseline) and days 4 to 9 identified no effect of day on saccharin/g body weight in IP-VEH mice ($p=0.20$; Fig. S1B). For IP-TNF20 mice there was a day effect ($F(6,42)=3.95$, $p<0.003$; Fig. S1C), with less saccharin/g body weight on day 5-7 and 9 versus baseline ($p<0.05$). For IP-TNF40 mice there was a day effect ($F(6,42)=14.62$, $p<0.0005$; Fig. S1D), with less saccharin/g body weight on days 4-7 and day 9 ($p<0.001$) compared to baseline.

Figure 3. Effects of 6 days i.p. injection on saccharin consumption

3.2. Osmotic micropump s.c. TNF delivery

3.2.1. Physiological effects

In a first cohort of mice, tail blood was collected on days 1, 3 and 6 and TNF plasma levels were determined. There was a group x day interaction ($F(2,39)=57.67$, $p<0.0005$; Fig. 4A), with plasma TNF levels increased in OP-TNF40 mice relative to OP-VEH mice at day 1 and to a lesser extent at day 3 and again at day 6. At day 7, compared to OP-VEH mice, OP-TNF40 mice had higher plasma levels of TNF (OP-VEH 0.75 ± 0.56, OP-TNF40 5.01 ± 1.18, $t(13)=9.46$, $p<0.0001$), IL-6 (OP-VEH 1.06 ± 0.24, OP-TNF40 3.10 ± 1.34, $t(12)=4.27$, $p<0.001$) and IL-10 (OP-VEH 0.74 ± 0.19, OP-TNF40 2.49 ± 1.53, $t(13)=3.44$, $p<0.005$). For absolute body weight, there was no group effect ($p\geq0.51$), whilst there was a main effect of day ($F(7,70)=2.46$, $p<0.03$) with body weight increasing as mice recovered post-surgery. For body weight at days 1-7 relative to day 0 baseline, there was a group x day interaction ($F(6,60)=2.89$, $p<0.02$; Fig. 4B): OP-TNF40 mice exhibited lower relative body weight than OP-VEH mice at day 1 specifically. On day 7, compared with OP-VEH mice, OP-TNF40 mice exhibited increased absolute spleen weight (OP-VEH 66.63 ± 6.49, OP-TNF40 109.00 ± 23.47, $t(10)=4.36$, $p<0.002$) and increased

spleen weight (mg) relative to body weight (g) (OP-VEH 2.10±0.18, OP-TNF40 3.47±0.68, $t(10)=4.78$, $p<0.0007$). Pronounced hemorrhagic lesions and fibrotic encapsulation were observed at the s.c. site of the pump delivery port in OP-TNF40 mice specifically.

3.2.2. Two-bottle saccharin test

In a separate cohort, mice implanted on day 0 with OP-TNF40 (n=6) or OP-VEH (n=6) pumps were studied in the two-bottle saccharin test. There was no effect of TNF on water consumption during days 1-5, and the two groups consumed a low and similar amount of water ($p\geq0.40$). For saccharin consumption there was a group x day interaction ($F(4,40)=3.72$, $p<0.05$; Fig. 4C): on day 2 OP-TNF40 mice consumed less saccharin than OP-VEH mice ($p<0.001$). In a posteriori group-specific analysis for days -3 (baseline) and 1 to 5, there was no day effect in OP-VEH mice ($p=0.07$; Fig. 4D). In OP-TNF40 mice there was a day effect ($F(5,25)=13.21$, $p<0.0005$; Fig. 4E), with decreased saccharin consumption on days 1 and 2 ($p<0.008$) and increased saccharin consumption on day 5 ($p<0.01$) compared to baseline. For saccharin preference there was a group x day interaction (($F(4,40)=2.76$, $p<0.04$): on day 5, OP-TNF40 mice (93±4 %) exhibited a higher saccharin preference than OP-VEH mice (82±17 %) ($p<0.04$). OP-TNF40 mice exhibited lower body weight than OP-VEH mice on day 1 specifically. The effects of OP-TNF on absolute saccharin consumption were also obtained when data were analyzed using saccharin consumed in g per g body weight (data not shown). The lower absolute saccharin consumption compared to that of mice in the IP-TNF study (Fig. 4 versus Fig. 3) could be attributable to reduced reward motivation related to surgery.

Figure 4. Effects of continuous peripheral delivery of TNF

3.3. Validation of central viral vector TNF expression

To determine the working titer for the scrAAV-8/2-CMV-chI-murine TNF vector, different titers were microinjected into medial hippocampus or amygdala. AAV-TNF at $1.1e^{10}$ vg/ml or $1.1e^{12}$ vg/ml or the control vector AAV-CON at $1.1e^{10}$ vg/ml were injected in 1µl unilaterally into the hippocampus. After 14 days, there was a titer-dependent increase in hippocampal TNF tissue concentration: 1.1e10 vg/ml yielded 3.2-100 pg TNF/mg protein (mean 23.6 pg/mg, Fig. 5A), an approximate 10-fold increase relative to mice injected with control AAV vector in which TNF levels were at or below the assay LLOQ i.e. < 0.3 pg/mg protein (Fig. 5A). Therefore, 1.1e10 vg/ml was used in the experiments where hippocampus or amygdala was the region of interest. Due to expected distribution to regions bordering the ventricles (see next paragraph), a higher titer, 1.1e11 vg/ml, was used for i.c.v. injection. In addition, Nissl staining was conducted to assess for effects of hippocampal viral vector TNF expression on cytoarchitecture. After 14 days of expression, mice were perfused transcardially and brains were sectioned (50 µm) and Nissl stained. Mice injected with the 1.1e10 vg/ml AAV-TNF vector in hippocampus demonstrated no detectable cytoarchitectural changes. In contrast, mice injected with 1.1e11 vg/ml or in particular 1.1e12 vg/ml titers exhibited altered cytoarchitecture consistent with gliosis and neurodegeneration in the injected hippocampus. To assess for biomarker evidence of functional AAV-TNF expression, the microglial protein, ionized calcium-binding adapter molecule 1 (Iba-1), was quantified in amygdala. Mice were injected in amygdala with AAV-TNF, AAV-CON or vehicle, and % area stained with Iba-1 was measured on day 14 (Fig. 5C and D). There was a group effect ($F(2,8)=29.86$, $p<0.001$; Fig. 5B), with higher Iba-1 expression in AAV-TNF mice compared to both AAV-CON ($p<0.001$) and VEH ($p<0.001$) mice, which had similar Iba-1 expression.

Figure 5. Validation of injection of a scrAAV-8/2-CMV-chI-murine TNF vector

To establish the brain regions that would likely incorporate the AAV-TNF vector subsequent to icv injection, AAV-EGFP was injected i.c.v. in two mice. After 14 days, EGFP expression was observed in the following regions: fimbria hippocampi (Fig. S2A), periventricular regions (surrounding the lateral and third ventricles, i.e. paraventricular thalamic nucleus, habenula, stria terminalis, dentate gyrus, and para-, supra and peri-ventricular hypothalamic nuclei; Fig. S2A), as well as hippocampus (Fig. S2B) and aqueduct (Fig. S2C).

3.4. ICV viral vector TNF expression

3.4.1. Physiological effects

In mice injected i.c.v. with AAV-TNF or AAV-CON and subsequently studied in behavioral tests, body weight was taken from injection at day 0 until day 22. Weekly-mean absolute body weights were similar in AAV-TNF and AAV-CON mice ($p \geq 0.43$), and increased in both groups across weeks ($p < 0.0005$). Also for body weight relative to day 0 baseline, there was no group effect ($p = 0.68$; Fig. 6B) and both groups increased in relative body weight across weeks ($F(2,48) = 19.80$, $p < 0.0005$). On day 22 brains were collected and, informed by the expression distribution in the AAV-EGFP experiment, levels of TNF and protein were determined in periventricular regions (hippocampus and aqueduct were not sampled). Tissue protein content was decreased in AAV-TNF mice (AAV-TNF 869±186 µg/ml, AAV-CON 1656±365 µg/ml, $t(18) = 6.61$, $p < 0.001$). TNF concentration was increased in AAV-TNF mice (0.22-0.95 pg/mg protein) compared to AAV-CON mice (TNF < LLOQ) ($t(17) = 2.27$, $p < 0.04$; Fig. 6A). In a separate cohort, effects of i.c.v. AAV-TNF expression on trunk blood plasma levels of TNF and IL-6 and IL-10 were assessed at 14 days post-injection; in AAV-TNF mice (n=6) and AAV-CON mice (n=3) plasma levels of each cytokine were below the assay LLOQ.

3.4.2. Contextual fear conditioning and memory

On day 14 post-injection, during a 5-min activity test in the arena, AAV-TNF and AAV-CON mice showed similar locomotor activity (AAV-CON 52363±7258 a.u., AAV-TNF 48246±7749 a.u., p=0.18), % time freezing (AAV-CON 0.98±1.60, AAV-TNF 1.08±0.84, p=0.84), and rearing (AAV-CON 15.33±6.04, AAV-TNF 19.92±5.99, p=0.07). Across six US trials, context conditioning was indicated by a main effect of trial (US ITI) on % time freezing ($F(2,46)=34.32$, $p<0.0005$; Fig. 6D). Furthermore, a main effect of group indicated more conditioning in AAV-TNF relative to AAV-CON mice ($F(1,34)=4.89$, $p<0.04$). US reactivity did not differ between AAV-TNF and AAV-CON mice (group main effect: p=0.45). On the following day, the two groups exhibited a similar % time freezing to the aversive context (group main effect: p=0.69; Fig. 6E). The main effect of interval ($F(5,115)=2.77$, $p<0.02$) reflected a general decline in freezing as the expression test progressed.

3.4.3. Two-way avoid-escape test

On day 16, a two-way avoid-escape test was conducted. Learning of the operant avoid-escape response was indicated by reduction in avoid-escape failures across trial blocks (CS-US trial main effect: $F(2,48)=12.54$, $p<0.0005$; Fig. 6F). The mean increase in avoid-escape failures by AAV-TNF mice was borderline non-significant (group main effect: p=0.09). For avoid responses per se there was also a borderline non-significant group main effect (p=0.06), with AAV-TNF mice making less avoid responses than AAV-CON mice. For % time freezing, which increased across trial blocks ($F(2,48)=45.16$, $p<0.0005$), there was a group x trial interaction ($F(2,48)=4.04$, $p<0.03$; Fig. 6G), with AAV-TNF mice freezing more at trials 11-20 than AAV-CON mice. For ITI compartment transfers there was a group x trial interaction ($F(2,48)=4.99$, $p<0.02$; Fig. 6H), with AAV-TNF mice transferring less than AAV-CON mice in trials 1-10. US reactivity did not differ between groups (p=0.45).

3.4.4. Two-bottle saccharin test

On days 18-19 mice were given two water bottles to assess AAV-TNF effects on water consumption; there was no effect (p=0.79). On test days 20-22, the two groups consumed low and similar amounts of water (group main effect: p=0.14), and high and similar amounts of saccharin (p=0.99; Fig. 6C); there was also no effect of group on saccharin preference (p=0.27). Similar findings were obtained using saccharin consumed in g per g body weight. The lower absolute consumption compared to that of mice in the IP-TNF study (Fig. 6 versus Fig. 3) could be attributable to the absence of pre-test exposure to saccharin in this experiment (and similarly in the amygdala TNF experiment, Fig. 8).

Figure 6. Long-term effects of bilateral i.c.v. injection of AAV-TNF

3.5. Hippocampal viral vector TNF expression

3.5.1. Physiological effects

In mice injected in the medial hippocampus with AAV-TNF or AAV-CON, there was no effect on mean-weekly absolute body weight (group main effect: p=0.31) and both groups increased in weight across weeks. For body weight relative to day 0 baseline, there was a main effect of group ($F(1,26)=5.06$, $p<0.04$; Fig. 7B) with lower relative body weight in AAV-TNF mice; relative body weight increased in both groups across weeks ($F(2,53)=18.43$, $p<0.005$). On day 16, following behavioral testing, brains were collected and levels of TNF and protein were determined in hippocampus. Tissue protein content was increased in AAV-TNF mice (AAV-TNF 2410±262 µg/ml, AAV-CON 1993.53±464 µg/ml, $t(15)=2.39$, $p<0.03$). The TNF concentration was increased in AAV-TNF mice (2.3-28.1 pg/mg) compared to AAV-CON mice (TNF < LLOQ of 0.3 pg/ml) ($t(15)=4.08$, $p<0.001$; Fig. 7A).

3.5.2. Contextual fear conditioning and memory

On day 14 post-injection, during the 5-min activity test, locomotor activity was lower in AAV-TNF mice (41217±8424 a.u.) compared to AAV-CON (52363±7258 a.u.) ($t(26)=13.47$, $p<0.001$), whereas % time freezing (AAV-CON 0.98±1.60, AAV-TNF 1.81±1.83, $p=0.22$) and rearing (AAV-CON 15.33±6.04, AAV-TNF 17.63±5.78, $p=0.32$), were similar in the two groups. Contextual conditioning was indicated by a main effect of trial (US ITI) on % time freezing ($F(2,54)=38.29$, $p<0.0005$; Fig. 7C). Furthermore, there was a group x trial interaction ($F(2,54)=3.78$, $p<0.03$), indicating increased acquisition of context freezing by AAV-TNF mice relative to AAV-CON mice during US-ITI 3-4 and 5-6. For US reactivity, there was a group x trial interaction ($F(2,54)=3.50$, $p<0.04$); at US 5-6, US reactivity was higher in AAV-TNF mice (770±183 a.u.) than AAV-CON mice (703±175) ($p<0.05$). Despite the greater acquisition of freezing on the previous day, in the context fear expression test the two groups exhibited similar % time freezing (group main effect: $p=0.59$, Fig. 7D). Percent time freezing in expression trials 1-2 was compared with that in conditioning trials 5-6 as an index of memory consolidation: freezing was reduced between acquisition and expression in AAV-TNF mice (paired t-test: $t(16)=2.22$, $p<0.05$) and not in AAV-CON mice ($p=0.80$) (Fig. 7E).

3.5.3. Two-way avoid-escape test

On day 16, in the two-way avoid-escape test, avoid-escape failures decreased across trial blocks (CS-US trial main effect: $F(2,52)=14.59$, $p<0.0005$; Fig. 7F) in the absence of a group effect ($p\geq0.83$). For avoids, there was a borderline non-significant main effect of group ($p=0.07$), with AAV-TNF mice tending to make less avoid responses than AAV-CON mice. Percent time freezing increased consistently across trial blocks ($F(2,52)=31.46$, $p<0.0005$; Fig. 7G). Furthermore, AAV-TNF mice exhibited more freezing than AAV-CON mice ($F(1,26)=7.37$, $p<0.02$). For ITI compartmental transfers there was a group x trial interaction ($F(2,52)=4.90$, $p<0.02$, Fig. 7H) and a group main effect ($F(1,26)=14.42$, $p<0.001$); AAV-TNF mice made less

ITI transfers than AAV-CON mice in each trial block. US reactivity did not differ between groups ($p=0.33$) and increased across trial blocks ($F(2,52)=8.81$, $p<0.001$).

Figure 7. Long-term effects of bilateral hippocampal injection of AAV-TNF

3.6. Amygdala viral vector TNF expression

3.6.1. Physiological effects

In mice injected in amygdala with AAV-TNF or AAV-CON, there was no effect on mean-weekly absolute body weight ($p=0.10$) and both groups increased in weight across weeks ($p<0.0005$). For body weight relative to day 0 baseline, there was no group effect ($p\geq0.15$; Fig. 8B); there was a main effect of week ($p<0.0005$) with both groups losing weight in week 1 followed by recovery. On day 22, following behavioral testing, TNF and protein levels were determined in amygdala. Tissue protein content was increased in AAV-TNF mice (AAV-TNF 1148±197 µg/ml, AAV-CON 926±225 µg/ml, $t(18)=2.33$, $p<0.03$). TNF concentration was increased in AAV-TNF (1.44-7.83 pg/mg protein) compared to AAV-CON mice (TNF < LLOQ of 0.3 pg/mL) ($t(18)=5.09$, $p<0.0001$; Fig. 8A). In a separate cohort, the effects of 14-day amygdala viral TNF expression on blood plasma TNF, IL-6 and IL-10 levels were assessed. Amygdala TNF was 4.57±0.92 pg/mg in AAV-TNF mice (n=6) and below the assay LLOQ in AAV-CON mice (n=3) ($t(7)=3.04$, $p<0.02$): in both groups, the plasma level of each cytokine was below the assay LLOQ. There was also no effect of amygdala AAV-TNF on spleen weight.

3.6.2. CS fear conditioning and memory

On day 14 post-injection, during the 5-min activity test, AAV-TNF and AAV-CON mice were similar in locomotor activity ($p=0.91$), % time freezing ($p=0.89$) and rearing ($p=1.00$). CS fear-conditioning in AAV-TNF and AAV-CON mice was indicated by the main effect of CS-US trial on % time freezing ($F(2,44)=23.46$, $p<0.0005$; Fig. 8D), with acquisition of CS freezing similar in the two groups (group effect: $p\geq0.41$). US reactivity did not differ between AAV-TNF and

AAV-CON mice (group effect: $p \geq 0.89$). On day 15, in the context fear expression test, maximum % time freezing (15-20%) occurred in minute 4-6 and declined in later intervals ($F(6,132)=5.20$, $p<0.0005$), and the two groups exhibited similar expression of freezing to context (group effect: $p \geq 0.45$) (data not shown). In the subsequent CS fear expression test, maximum % time freezing occurred at CS 1-2 and then declined ($F(5,110)=6.97$, $p<0.0005$; Fig. 8E), and the two groups exhibited similar expression of freezing to the CS (group effect: $p \geq 0.94$).

3.6.3. Two-way escape test

On day 16, in the two-way escape test, operant learning of the escape response was indicated by reduction in escape failures across trial blocks ($F(2,44)=4.49$, $p<0.02$; Fig. 8F), with the two groups not differing on this measure (group main effect: $p=0.18$). Percent time freezing increased minimally but consistently across trials ($F(2,44)=8.07$, $p<0.001$; Fig. 8G), with no effect of group ($p=0.32$). AAV-TNF mice made less ITI transfers than AAV-CON mice (main effect: ($F(1,22)=8.74$, $p<0.01$; Fig. 8H) and ITI transfers were consistent across ITI blocks ($p=0.64$). US reactivity did not differ between groups ($p=0.22$) and was consistent across trial blocks ($p=0.26$).

3.6.4. Two-bottle saccharin test

On days 18-19 mice were given two water bottles to assess AAV-TNF effects on water consumption: AAV-TNF mice drank more water than AAV-CON mice on each of the two days (group main effect: $F(1,22)=8.11$, $p<0.01$). On days 20-22, water consumption, saccharin consumption and saccharin preference were measured. Across days, the two groups consumed a low and similar amount of water (group main effect: $p=0.13$). For saccharin consumption there was a group x day interaction ($F(2,44)=4.91$, $p=0.02$; Fig. 8C), with AAV-TNF mice consuming less saccharin than AAV-CON mice on the second test day. For

saccharin preference there was no effect of group (p=0.37) or day (p=1.00). This effect was also obtained when the data were analyzed using saccharin consumed in g per g body weight.

Figure 8. Long-term effects of bilateral amygdala injection of AAV-TNF

4. Discussion

Tumor necrosis factor is associated with sickness and depression in humans and animal models. Because it is expressed in the periphery and the brain, and can also transfer between these compartments, it is important but challenging to establish the specific substrates at which TNF exerts its diverse effects. In mice, by investigating the effects of increased TNF on immune-inflammation, sickness and emotional and cognitive behavior in the periphery or distinct brain regions specifically, the present study adds substantially to the understanding of the psychoneuroimmunology of TNF effects.

4.1. Effects of peripheral TNF

It was not possible to induce a peripheral TNF increase that was continuous and maintained for 7-10 days. Using TNF injection (IP) for 10 days, there was a daily acute high level of plasma TNF, whilst osmotic micropump (OP) delivery achieved a continuous plasma TNF increase that declined across days related to hemorrhagic necrosis at the pump outlet as reported previously (Cavadini et al., 2007). (Customized s.c. pellets coated with TNF (Innovative Research of America) were ineffective in increasing plasma TNF beyond 1 day (unpublished data)). Despite these methodological constraints, IP- and OP-TNF resulted in consistent and similar effects. Thus, IP-TNF led to sickness in the form of reduced body weight, and this co-occurred with reduced saccharin consumption on days 4-7 and increased

memory for an aversive conditioned stimulus on days 4-5. In OP-TNF mice the sickness effect was less marked, but saccharin consumption was again reduced, on days 1-2. These IP- and OP-TNF effects co-occurred with increases in plasma levels of IL-6 and IL-10 and in spleen weight, indicating that peripheral TNF stimulated immune-inflammatory response pathways.

Previous mouse studies used i.p. challenge to stimulate generalized immune-inflammation including increased plasma TNF. Bacterial LPS stimulates toll-like receptor 4 and induces increased TNF and, among others, IFN-γ, IL-1β and IL-6, in the immune compartment, blood and brain. These cytokine increases co-occur with acute sickness, including loss of body weight, peaking at 4-6 h and subsiding by about 24 h post-LPS. There is a 1-2 day decrease in preference for and/or absolute consumption of sucrose or saccharin (Aubert and Dantzer, 2005; Biesmans et al., 2013; Salazar et al., 2012). Learning-memory studies have focused on contextual fear conditioning and with LPS injected directly after conditioning: fear expression was decreased indicating impaired consolidation of context memory (Dantzer et al., 2008; Pugh et al., 1998; Thomson and Sutherland, 2005). Agonist antibody for TNF receptor superfamily member CD40 (CD40AB) induces, in parallel to multi-organ inflammation, lymphadenopathy and splenomegaly, increased blood TNF and blood and brain IFN-γ. Sickness occurs for 2-3 days and saccharin drinking is decreased for 7 days. These CD40AB effects are prevented by co-administration of TNF inhibitor (etanercept). At day 5 after CD40AB, CS fear learning is impaired, in the absence of an additional memory deficit at day 6 (Cathomas et al., 2015b; Gast et al., 2013).

Integrating the present IP-/OP-TNF findings with those of the peripheral LPS and CD40AB studies, there are consistent effects on sickness and reward drinking, and the present study adds to the evidence for the prominent involvement of peripheral TNF as a trigger in the causation of these effects. However, whilst IP-TNF increased CS fear memory,

LPS impairs context fear memory and CD40AB impairs CS fear learning without an additional effect on memory. These findings indicate that when TNF alone is the inflammatory trigger, the downstream changes lead to increased consolidation of CS-US learning and presumably involve the amygdala (see section 4.5). In contrast, when a more pronounced and heterogeneous trigger is involved, this TNF effect is masked by downstream changes that lead to impaired learning-memory.

Peripheral TNF tended to increase TNF levels in brain regions bordering the 3rd, 4th and lateral ventricles (referred to as periventricular regions) at 1 h post-injection. Using IP human TNF, we could provide supportive evidence for TNF peripheral-brain passage: human and murine TNF protein structures are similar (Brouckaert et al., 1986; Kuprash et al., 1999) and should exhibit similar peripheral-brain passage (Banks et al., 1995), although human TNF exhibits higher binding to TNF receptor 1 (Ameloot et al., 2001) which might enhance its passage. A single ip TNF injection increased whole brain *Tnf* mRNA and TNF protein expression at 1 h post-injection (Qin et al., 2007). A single intravenous TNF injection induced increased immediate-early gene (*c-Fos*) expression in the circumventricular organs (Nadeau and Rivest, 1999); the latter express TNF receptors, suggesting that peripheral TNF enters this region and contributes to changes in brain function. Also highly relevant here is the evidence that i.c.v. TNF is, together with IL-1β, the major trigger of sickness (for review: Dantzer and Kelley, 2007), a point to which we return when discussing the i.c.v. AAV-TNF findings (see section 4.3). Peripheral TNF did not lead to increased TNF level in the hippocampus or amygdala. Although not considered in this study, centrally-processed manipulations that activate peripheral inflammation, including psychosocial stress, result in increased recruitment of macrophages to the brain (e.g. (Wohleb et al., 2013)). T-cell activation and consequent cytokine release and passage, e.g. IL-2, is another candidate

mechanism via which peripheral immune-inflammation can influence brain state (Kammula et al., 1998; Miller et al., 2009; Mössner et al., 2007).

4.2. AAV-vector brain TNF expression

Using a TNF scAAV vector, a moderate and brain region-specific increase in TNF that was continuous and maintained for at least 3-4 weeks was induced. AAV vectors derived from serotype 2 transduce neurons and glia efficiently, in the absence of obvious cell damage in the brain tissue (Paterna et al., 2000, 2004; Fu et al., 2003). Using AAV2-ITR-based genomic constructs packaged into capsids of AAV serotype 8, efficient transduction of all major brain cell types was demonstrated, being highest in astrocytes (Aschauer et al., 2013). Protein expression was observed at day 3 after scAAV injection into mouse brain, with a steady-state proportion of transduced cells observed at day 8, and the amount of expressed protein increasing up to day 21 (Aschauer et al., 2013). Our pilot studies allowed for the identification of a TNF scAAV vector titer that yielded a moderate increase in TNF level, was specific to the region of interest, and did not induce gliosis or neurodegeneration, but did induce local microglial activation. AAV control vector and vehicle control mice expressed similar, basal levels of Iba-1 expression in the injection region and the AAV-CON vector did not induce TNF expression, in agreement with the evidence that AAV-vectors do not induce immunogenic activation (Aschauer et al., 2013; Howard et al., 2008; McCarthy et al., 2008; McCarty et al., 2001). An additional general finding was that our assay for total protein identified a decrease in periventricular regions using ICV AAV-TNF at 1.1e11 vg/ml and an increase in total protein in amygdala and hippocampus using ICV AAV-TNF at 1.1e10 vg/ml; the former decrease might reflect moderate neurodegeneration and the latter increases might well reflect recruitment of immune cells in line with the increased activation of microglia. Given the findings of this study, but also accepting that the effects of increased

TNF expression in the brain might well be dependent on the cell types expressing it, subsequent studies should investigate the effects of brain region-specific increases in *Tnf* expression achieved using vectors that result in cell type-specific expression.

4.3. Effects of ICV TNF

Acute i.c.v. injection of TNF leads to sickness, and decreases in social exploration, active responding in the forced swim and tail suspension tests, and in sucrose consumption (Bluthé et al., 1994; Palin et al., 2007, 2009; Kaster et al., 2012). In the peripheral TNF experiments the increase in periventricular-region TNF was likely due to passage through the relatively permeable ventricle interfaces of the blood-brain barrier (Fu et al., 2003; Ueno et al., 2000). Using AAV-EGFP, ICV injection resulted in viral transduction and stable, long-term expression in these same periventricular regions, as well as in hippocampus and aqueduct. Using ICV AAV-TNF, at 3-4 weeks post-injection there was a mild increase in TNF in the periventricular regions; these regions express TNF receptor 1 (Nadeau and Rivest, 1999). This occurred in the absence of an increase in plasma TNF (or other cytokines). This allowed for the study of the effects of a specific, chronic increase in periventricular-region TNF on sickness and depression- and anxiety disorder-relevant behavior. Two important differences to the IP-TNF experiment and to previous studies of acute i.c.v. TNF were the absences of a sickness effect in terms of reduced body weight and of a reward-interest effect in terms of reduced saccharin drinking. Integration of these findings indicates that increased periventricular-region TNF is not sufficient to induce sickness or reduce reward consumption, and also suggests that these two behavioral effects are inter-dependent i.e. reduced reward drinking follows sickness. In the context fear conditioning experiment, ICV AAV-TNF mice exhibited increased acquisition of fear-freezing by the end of the conditioning session, consistent with increased salience of the aversive environment, although this did not translate into an

increase in contextual fear memory. In the two-way avoid-escape test, freezing was also increased, together with reduced compartmental transfers, again consistent with increased salience of the aversive environment. One of the regions transduced by ICV AAV vector was the periaqueductal grey (PAG), which is a major mediator of the freezing response (Kim et al., 1993; Kim et al., 2013).

4.4. Effects of hippocampal TNF

The region-specific and sustained increases in TNF attainable with the AAV-vector allowed for the first experiments on the effects of a specific increase in hippocampal TNF or amygdala TNF. The hippocampus expresses TNF receptor 1 under basal conditions (Bette et al., 2003). It is a region of major importance in spatial and contextual learning-memory (dorsal hippocampus) as well as in the processing of the active approach-passive avoidance conflict that underlies anxiety (ventral hippocampus) (Maren et al., 2013; Orsini and Maren, 2012; Bannerman et al., 2014); furthermore, ventral hippocampus projects to nucleus accumbens (Britt et al., 2012), a major region in the circuitry of reward processing. AAV-TNF was injected and transduced in the medial hippocampus. In contrast to ICV AAV-TNF, hippocampal AAV-TNF induced sickness, possibly related to a greater increase in hippocampal TNF following direct injection. At the activity-test phase of contextual fear conditioning, AAV-TNF mice exhibited decreased locomotion, possibly indicative of increased hippocampal inhibition of nucleus accumbens-modulated exploration (Britt et al., 2012). These same mice exhibited increased acquisition of fear-freezing across the conditioning session, consistent with increased salience of the aversive environment, as observed with ICV AAV-TNF. In addition, hippocampal AAV-TNF mice exhibited a decrease in contextual fear expression relative to acquisition, consistent with inhibition of consolidation and/or recall of contextual memory. Interestingly, a similar deficit in contextual fear memory was

observed in a mouse model of experimental autoimmune encephalomyelitis (EAE): the deficit co-occurred with a moderate increase in hippocampal TNF and was mediated by the TNF receptor 1 expressed on astrocytes (Habbas et al., 2015). Further evidence that increased hippocampal TNF increases sensitivity to aversive stimuli was obtained in the two-way avoid-escape test: as for ICV AAV-TNF mice, mice expressing increased hippocampal TNF exhibited increased freezing together with decreased compartmental transfers; these changes suggest increased anxiety and, therefore, that TNF increases the approach-avoidance conflict and resultant behavioral inhibition that has been attributed to ventral hippocampus in such ambivalent environments (Bannerman et al., 2014; McNaughton and Gray, 2000). The direct evidence for anxiogenic effects of hippocampal TNF adds to that from immune challenge studies where cytokine increases, including hippocampal *Tnf*, lead to increased anxiety (Silverman et al., 2006).

4.5. Effects of amygdala TNF

Under basal conditions, the amygdala does not express TNF receptor 1 (Bette et al., 2003; Nadeau and Rivest, 1999). Nonetheless, AAV-TNF functionality in the amygdala was demonstrated by a marked, local activation of microglia. Amygdala AAV-TNF was without effect on body weight, but it had a transient effect in terms of reduced saccharin drinking. As for ventral hippocampus, the amygdala projects to the nucleus accumbens (Britt et al., 2012) and this is one of the pathways via which amygdala function can modulate reward-directed behavior (Der-Avakian and Markou, 2012). The amygdala is best-studied in terms of its regulation of classical conditioning to aversive stimuli (Duvarci and Pare, 2014; Johansen et al., 2011; Phelps and LeDoux, 2005). Increased amygdala TNF expression was without effect on either acquisition of CS-US fear-freezing or the memory processes determining its next-day expression. A recent rat study found that an acute injection of TNF into the amygdala led

on the next day to reduced acquisition and also impaired extinction of aversive CS-US conditioning (Jing et al., 2015). In a mouse EAE model, the CS memory was reduced, but this occurred in the absence of increased amygdala TNF, indicating that other immune-inflammation factors were responsible (Acharjee et al., 2013). In the two-way escape test, amygdala AAV-TNF mice made less compartment transfers indicating an increase in conflict anxiety, consistent with a contribution of the amygdala to this behavioral state (McNaughton and Gray, 2000).

4.6. Integration and implications

The present mouse model study provides important new insights into the distinct roles of increases in TNF in the circulation and in specific brain regions on well-being and depression- and anxiety disorder-relevant behavior. It highlights the complexity in terms of the indirect and direct pathways via which increases in TNF can act and which need to be taken into account when considering it as a target for therapy in mental disorders. Increased TNF in the circulation resulted in increased TNF in the periventricular regions, as did injection of an AAV vector expressing TNF into the lateral ventricles. The same vector also became locally transduced and resulted in a moderate increase in TNF level and in microglia activation, when injected into specific brain regions.

Sickness describes the condition induced by immune-inflammation associated with pathogenic infections, autoimmune disorders and cytokine-based therapies. It comprises heterogeneous states including decreased appetite, weight loss, malaise, cognitive impairment and fatigue (for review: (Dantzer et al., 2008)). Using sustained body weight loss as a marker, sickness was induced by IP TNF and hippocampal TNF, but not by TNF in periventricular regions or amygdala. Reduced interest in rewarding events/stimuli, often associated with weight loss, is a core symptom in depression and a dimension in the RDoC

domain Positive valence systems (American Psychiatric Association, 2013; Cuthbert and Insel, 2013). There is interest in the inter-relationship between reduced reward interest and the decreased appetite/weight loss of sickness with respect to there being a common pathophysiology (Dantzer et al., 2008; Raison et al., 2013). Immune-inflammatory challenges such as LPS and CD40AB induce sickness including weight loss, and co-occurring with this and extending beyond it is a reduction in reward drinking (Aubert and Dantzer, 2005; Biesmans et al., 2013; Cathomas et al., 2015b; Salazar et al., 2012). The findings that IP TNF induces sickness and reduces saccharin drinking add to the evidence that these two states co-occur. Previously, we have proposed that under these conditions reduced reward drinking is more parsimoniously referred to as extended sickness (Cathomas et al., 2015b; Pryce and Fontana, 2016). This is because immune-inflammatory challenges produce a different constellation of behavioral changes e.g. increased sensitivity to the bitter component of saccharin (Aubert and Dantzer, 2005), reduced interest in gustatory reward (saccharin, sucrose) (Cathomas et al., 2015b; Salazar et al., 2012), decreased fear learning-memory (Cathomas et al., 2015b; Dantzer et al., 2008; Thomson and Sutherland, 2005), than do psychosocial stressors e.g. reduced interest in gustatory reward (Bergamini et al., 2016; Willner, 1997), increased fear learning-memory (Fuertig et al., 2016). Accordingly, it should not be assumed that the pathophysiologies underlying shared behavioral states, e.g. reduced interest in gustatory reward, are the same. Nonetheless, that amygdala TNF reduced saccharin consumption in the absence of sickness provides some of the first evidence that central inflammation impacts on reward processing in the absence of sickness.

Emotions and motivations induced by aversive stimuli are dimensions in the RDoC domain Negative valence systems, with the most important being fear and anxiety (Cuthbert and Insel, 2013). As demonstrated with the fear learning-memory tests, both periventricular

and hippocampal TNF increase learning about an aversive context, whilst hippocampal TNF decreases the memory of this context. IP TNF was without effect on learning about an aversive CS but increased the memory of this CS, and amygdala TNF did not impact on either learning about or memory of an aversive CS. Whilst adding to the evidence that TNF is an important inhibitor of hippocampal memory function, the present study also indicate that this does not extend to amygdala memory function. With respect to anxiety induced by approach-avoidance conflict, the effects of central increases in TNF were rather more consistent, with each of paraventricular, hippocampal and amygdala TNF leading to an increase in anxiety on at least one measure of the test. Overall with respect to effects on aversive stimulus processing, this study provides robust evidence for TNF-mediated increased aversive reactivity, as well as adding to the evidence for impairment of memory.

This study of the effects of specific increases in TNF levels on inflammation, sickness, emotional behavior and memory in mice adds to the understanding of the importance of TNF for each of these states. Furthermore, the findings demonstrate that moderate TNF increases in peripheral or specific brain regions exert distinctive effects on emotional and cognitive processes that are disturbed in depression and anxiety disorders, as well as in immune disorders presenting with psychopathologies. Given that peripheral and central increases in TNF can occur independently of each other, the evidence is that the current focus on neutralizing peripheral increases in TNF will need to be complemented by treatments targeting central TNF, if highly efficacious TNF-based therapies are to be investigated.

Acknowledgments

We are grateful to Björn Henz and Ramiro Valero for support in animal maintenance, to

Emese Szabo and Melanie Rauch for expert technical support in vector cloning, and to Jean-Marc Fritschy for advice on immunohistochemistry procedures. This research was funded by the Swiss National Science Foundation (SNSF; grant 31003A-141137 to C.R.P. and E.S.). F.K. received a MD-PhD fellowship from the SNSF (323530-151482). Adriano Fontana is a Hertie Senior Research Professor for Neuroscience of the Gemeinnützige Hertie-Stiftung.

References

Acharjee, S., Nayani, N., Tsutsui, M., Hill, M.N., Ousman, S.S., and Pittman, Q.J. (2013). Altered cognitive-emotional behavior in early experimental autoimmune encephalitis – Cytokine and hormonal correlates. Brain. Behav. Immun. *33*, 164–172.

Ameloot, P., Fiers, W., Bleser, P.D., Ware, C.F., Vandenabeele, P., and Brouckaert, P. (2001). Identification of Tumor Necrosis Factor (TNF) Amino Acids Crucial for Binding to the Murine p75 TNF Receptor and Construction of Receptor-selective Mutants. J. Biol. Chem. *276*, 37426–37430.

American Psychiatric Association (2013). Diagnostical and Statistical Manual of Mental Disorders: DSM-V-TR: 5th Edition (Washington, DC: American Psychiatric Association).

Aschauer, D.F., Kreuz, S., and Rumpel, S. (2013). Analysis of Transduction Efficiency, Tropism and Axonal Transport of AAV Serotypes 1, 2, 5, 6, 8 and 9 in the Mouse Brain. PLoS ONE *8*, e76310.

Aubert, A., and Dantzer, R. (2005). The taste of sickness: Lipopolysaccharide-induced finickiness in rats. Physiol. Behav. *84*, 437–444.

Azzinnari, D., Sigrist, H., Staehli, S., Palme, R., Hildebrandt, T., Leparc, G., Hengerer, B., Seifritz, E., and Pryce, C.R. (2014). Mouse social stress induces increased fear conditioning, helplessness and fatigue to physical challenge together with markers of altered immune and dopamine function. Neuropharmacology *85*, 328–341.

Baens, M., Noels, H., Broeckx, V., Hagens, S., Fevery, S., Billiau, A.D., Vankelecom, H., and Marynen, P. (2006). The Dark Side of EGFP: Defective Polyubiquitination. PLoS ONE *1*, e54.

Banks, W.A., Plotkin, S.R., and Kastin, A.J. (1995). Permeability of the Blood-Brain Barrier to

Soluble Cytokine Receptors. Neuroimmunomodulation *2*, 161–165.

Bannerman, D.M., Sprengel, R., Sanderson, D.J., McHugh, S.B., Rawlins, J.N.P., Monyer, H., and Seeburg, P.H. (2014). Hippocampal synaptic plasticity, spatial memory and anxiety. Nat. Rev. Neurosci. *15*, 181–192.

Bergamini, G., Cathomas, F., Auer, S., Sigrist, H., Seifritz, E., Patterson, M., Gabriel, C., and Pryce, C.R. (2016). Mouse psychosocial stress reduces motivation and cognitive function in operant reward tests: A model for reward pathology with effects of agomelatine. Eur. Neuropsychopharmacol. *in press.*

Bette, M., Kaut, O., Schäfer, M.K.-H., and Weihe, E. (2003). Constitutive expression of p55TNFR mRNA and mitogen-specific up-regulation of TNFα and p75TNFR mRNA in mouse brain. J. Comp. Neurol. *465*, 417–430.

Biesmans, S., Meert, T.F., Bouwknecht, J.A., Acton, P.D., Davoodi, N., De Haes, P., Kuijlaars, J., Langlois, X., Matthews, L.J.R., Ver Donck, L., et al. (2013). Systemic Immune Activation Leads to Neuroinflammation and Sickness Behavior in Mice, Systemic Immune Activation Leads to Neuroinflammation and Sickness Behavior in Mice. Mediat. Inflamm. Mediat. Inflamm. *2013*, *2013*, e271359.

Bluthé, R.M., Pawlowski, M., Suarez, S., Parnet, P., Pittman, Q., Kelley, K.W., and Dantzer, R. (1994). Synergy between tumor necrosis factor α and interleukin-1 in the induction of sickness behavior in mice. Psychoneuroendocrinology *19*, 197–207.

Bluthé, R.-M., Michaud, B., Poli, V., and Dantzer, R. (2000). Role of IL-6 in cytokine-induced sickness behavior: a study with IL-6 deficient mice. Physiol. Behav. *70*, 367–373.

Britt, J.P., Benaliouad, F., McDevitt, R.A., Stuber, G.D., Wise, R.A., and Bonci, A. (2012). Synaptic and Behavioral Profile of Multiple Glutamatergic Inputs to the Nucleus Accumbens. Neuron *76*, 790–803.

Brouckaert, P.G.G., Leroux-Roels, G.G., Guisez, Y., Tavernier, J., and Fiers, W. (1986). In vivo anti-tumour activity of recombinant human and murine TNF, alone and in combination with murine IFN-γ, on a syngeneic murine melanoma. Int. J. Cancer *38*, 763–769.

Cathomas, F., Stegen, M., Sigrist, H., Schmid, L., Seifritz, E., Gassmann, M., Bettler, B., and Pryce, C.R. (2015a). Altered emotionality and neuronal excitability in mice lacking KCTD12, an auxiliary subunit of GABAB receptors associated with mood disorders. Transl. Psychiatry *5*, e510.

Cathomas, F., Fuertig, R., Sigrist, H., Newman, G.N., Hoop, V., Bizzozzero, M., Mueller, A., Luippold, A., Ceci, A., Hengerer, B., et al. (2015b). CD40-TNF activation in mice induces extended sickness behavior syndrome co-incident with but not dependent on activation of the kynurenine pathway. Brain. Behav. Immun.

Cavadini, G., Petrzilka, S., Kohler, P., Jud, C., Tobler, I., Birchler, T., and Fontana, A. (2007). TNF-α suppresses the expression of clock genes by interfering with E-box-mediated transcription. Proc. Natl. Acad. Sci. *104*, 12843–12848.

Cuthbert, B.N., and Insel, T.R. (2013). Toward the future of psychiatric diagnosis: the seven pillars of RDoC. BMC Med. *11*, 126.

Dantzer, R., O'Connor, J.C., Freund, G.G., Johnson, R.W., and Kelley, K.W. (2008). From inflammation to sickness and depression: when the immune system subjugates the brain. Nat Rev Neurosci *9*, 46–56.

Dean, B., Tawadros, N., Scarr, E., and Gibbons, A.S. (2010). Regionally-specific changes in levels of tumour necrosis factor in the dorsolateral prefrontal cortex obtained postmortem from subjects with major depressive disorder. J. Affect. Disord. *120*, 245–248.

Der-Avakian, A., and Markou, A. (2012). The neurobiology of anhedonia and other reward-

related deficits. Trends Neurosci. *35*, 68–77.

Dowlati, Y., Herrmann, N., Swardfager, W., Liu, H., Sham, L., Reim, E.K., and Lanctôt, K.L. (2010). A meta-analysis of cytokines in major depression. Biol. Psychiatry *67*, 446–457.

DuBridge, R.B., Tang, P., Hsia, H.C., Leong, P.M., Miller, J.H., and Calos, M.P. (1987). Analysis of mutation in human cells by using an Epstein-Barr virus shuttle system. Mol. Cell. Biol. *7*, 379–387.

Duvarci, S., and Pare, D. (2014). Amygdala Microcircuits Controlling Learned Fear. Neuron *82*, 966–980.

Erickson, M.A., and Banks, W.A. (2011). Cytokine and chemokine responses in serum and brain after single and repeated injections of lipopolysaccharide: Multiplex quantification with path analysis. Brain. Behav. Immun. *25*, 1637–1648.

Frenois, F., Moreau, M., O'Connor, J., Lawson, M., Micon, C., Lestage, J., Kelley, K.W., Dantzer, R., and Castanon, N. (2007). Lipopolysaccharide induces delayed FosB/DeltaFosB immunostaining within the mouse extended amygdala, hippocampus and hypothalamus, that parallel the expression of depressive-like behavior. Psychoneuroendocrinology *32*, 516–531.

Fu, H., Muenzer, J., Samulski, R.J., Breese, G., Sifford, J., Zeng, X., and McCarty, D.M. (2003). Self-Complementary Adeno-associated Virus Serotype 2 Vector: Global Distribution and Broad Dispersion of AAV-Mediated Transgene Expression in Mouse Brain. Mol. Ther. *8*, 911–917.

Fuertig, R., Azzinnari, D., Bergamini, G., Cathomas, F., Sigrist, H., Seifritz, E., Vavassori, S., Luippold, A., Hengerer, B., Ceci, A., et al. (2016). Mouse chronic social stress increases blood and brain kynurenine pathway activity and fear behaviour: Both effects are reversed by inhibition of indoleamine 2,3-dioxygenase. Brain. Behav. Immun. *54*, 59–72.

Gao, G.-P., Alvira, M.R., Wang, L., Calcedo, R., Johnston, J., and Wilson, J.M. (2002). Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy. Proc. Natl. Acad. Sci. *99*, 11854–11859.

Gast, H., Müller, A., Lopez, M., Meier, D., Huber, R., Dechent, F., Prinz, M., Emmenegger, Y., Franken, P., Birchler, T., et al. (2013). CD40 activation induces NREM sleep and modulates genes associated with sleep homeostasis. Brain. Behav. Immun. *27*, 133–144.

Graham, F.L., Smiley, J., Russell, W.C., and Nairn, R. (1977). Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol. *36*, 59–74.

Habbas, S., Santello, M., Becker, D., Stubbe, H., Zappia, G., Liaudet, N., Klaus, F.R., Kollias, G., Fontana, A., Pryce, C.R., et al. (2015). Neuroinflammatory TNFα Impairs Memory via Astrocyte Signaling. Cell *163*, 1730–1741.

Haji, N., Mandolesi, G., Gentile, A., Sacchetti, L., Fresegna, D., Rossi, S., Musella, A., Sepman, H., Motta, C., Studer, V., et al. (2012). TNF-α-mediated anxiety in a mouse model of multiple sclerosis. Exp. Neurol. *237*, 296–303.

van Heesch, F., Prins, J., Korte-Bouws, G.A.H., Westphal, K.G.C., Lemstra, S., Olivier, B., Kraneveld, A.D., and Korte, S.M. (2013). Systemic tumor necrosis factor-alpha decreases brain stimulation reward and increases metabolites of serotonin and dopamine in the nucleus accumbens of mice. Behav. Brain Res. *253*, 191–195.

Howard, D.B., Powers, K., Wang, Y., and Harvey, B.K. (2008). Tropism and toxicity of adeno-associated viral vector serotypes 1, 2, 5, 6, 7, 8, and 9 in rat neurons and glia in vitro. Virology *372*, 24–34.

Jing, H., Hao, Y., Bi, Q., Zhang, J., and Yang, P. (2015). Intra-amygdala microinjection of TNF-α impairs the auditory fear conditioning of rats via glutamate toxicity. Neurosci. Res. *91*, 34–40.

Johansen, J.P., Cain, C.K., Ostroff, L.E., and LeDoux, J.E. (2011). Molecular Mechanisms of Fear Learning and Memory. Cell *147*, 509–524.

Kammula, U.S., White, D.E., and Rosenberg, S.A. (1998). Trends in the safety of high dose bolus interleukin-2 administration in patients with metastatic cancer. Cancer *83*, 797–805.

Kaster, M.P., Gadotti, V.M., Calixto, J.B., Santos, A.R.S., and Rodrigues, A.L.S. (2012). Depressive-like behavior induced by tumor necrosis factor-α in mice. Neuropharmacology *62*, 419–426.

Kim, J.J., Rison, R.A., and Fanselow, M.S. (1993). Effects of amygdala, hippocampus, and periaqueductal gray lesions on short- and long-term contextual fear. Behav. Neurosci. *107*, 1093–1098.

Kinsey, S.G., Bailey, M.T., Sheridan, J.F., and Padgett, D.A. (2008). The inflammatory response to social defeat is increased in older mice. Physiol. Behav. *93*, 628–636.

Kuprash, D.V., Udalova, I.A., Turetskaya, R.L., Kwiatkowski, D., Rice, N.R., and Nedospasov, S.A. (1999). Similarities and differences between human and murine TNF promoters in their response to lipopolysaccharide. J. Immunol. *162*, 4045–4052.

Lawson, M.A., McCusker, R.H., and Kelley, K.W. (2013). Interleukin-1 beta converting enzyme is necessary for development of depression-like behavior following intracerebroventricular administration of lipopolysaccharide to mice. J. Neuroinflammation *10*, 54.

Maes, M. (1995). Evidence for an immune response in major depression: A review and hypothesis. Prog. Neuropsychopharmacol. Biol. Psychiatry *19*, 11–38.

Maren, S., Phan, K.L., and Liberzon, I. (2013). The contextual brain: implications for fear conditioning, extinction and psychopathology. Nat. Rev. Neurosci. *14*, 417–428.

Marques-Vidal, P., Bochud, M., Bastardot, F., Lüscher, T., Ferrero, F., Gaspoz, J.-M., Paccaud, F., Urwyler, A., von Känel, R., Hock, C., et al. (2011). Levels and Determinants of Inflammatory Biomarkers in a Swiss Population-Based Sample (CoLaus Study). PLoS ONE *6*.

McCarthy, M.I., Abecasis, G.R., Cardon, L.R., Goldstein, D.B., Little, J., Ioannidis, J.P.A., and Hirschhorn, J.N. (2008). Genome-wide association studies for complex traits: consensus, uncertainty and challenges. Nat. Rev. Genet. *9*, 356–369.

McCarty, D.M., Monahan, P.E., and Samulski, R.J. (2001). Self-complementary recombinant adeno-associated virus (scAAV) vectors promote efficient transduction independently of DNA synthesis. Gene Ther. *8*, 1248–1254.

McCarty, D.M., Fu, H., Monahan, P.E., Toulson, C.E., Naik, P., and Samulski, R.J. (2003). Adeno-associated virus terminal repeat (TR) mutant generates self-complementary vectors to overcome the rate-limiting step to transduction in vivo. Gene Ther. *10*, 2112–2118.

McNaughton, N., and Gray, J.A. (2000). Anxiolytic action on the behavioural inhibition system implies multiple types of arousal contribute to anxiety. J. Affect. Disord. *61*, 161–176.

Miller, A.H., Maletic, V., and Raison, C.L. (2009). Inflammation and Its Discontents: The Role of Cytokines in the Pathophysiology of Major Depression. Biol. Psychiatry *65*, 732–741.

Mössner, R., Mikova, O., Koutsilieri, E., Saoud, M., Ehlis, A.-C., Müller, N., Fallgatter, A.J., and Riederer, P.D.P. (2007). Consensus paper of the WFSBP Task Force on Biological Markers: Biological Markers in Depression. World J. Biol. Psychiatry *8*, 141–174.

Murray, C.J.L., and Lopez, A.D. (1996). Evidence-based health policy--lessons from the Global Burden of Disease Study. Science *274*, 740–743.

Nadeau, S., and Rivest, S. (1999). Effects of circulating tumor necrosis factor on the neuronal activity and expression of the genes encoding the tumor necrosis factor receptors (p55 and p75) in the rat brain: a view from the blood–brain barrier. Neuroscience *93*, 1449–1464.

Orsini, C.A., and Maren, S. (2012). Neural and Cellular Mechanisms of Fear and Extinction Memory Formation. Neurosci. Biobehav. Rev. *36*, 1773–1802.

Palin, K., Bluthé, R.-M., McCusker, R.H., Moos, F., Dantzer, R., and Kelley, K.W. (2007). TNFα-induced sickness behavior in mice with functional 55 kD TNF receptors is blocked by central IGF-I. J. Neuroimmunol. *187*, 55–60.

Palin, K., Bluthé, R.-M., McCusker, R.H., Levade, T., Moos, F., Dantzer, R., and Kelley, K.W. (2009). The type 1 TNF receptor and its associated adapter protein, FAN, are required for TNFα-induced sickness behavior. Psychopharmacology (Berl.) *201*, 549–556.

Paterna, J.C., Moccetti, T., Mura, A., Feldon, J., and Büeler, H. (2000). Influence of promoter and WHV post-transcriptional regulatory element on AAV-mediated transgene expression in the rat brain. Gene Ther. *7*, 1304–1311.

Paterna, J.-C., Feldon, J., and Büeler, H. (2004). Transduction Profiles of Recombinant Adeno-Associated Virus Vectors Derived from Serotypes 2 and 5 in the Nigrostriatal System of Rats. J. Virol. *78*, 6808–6817.

Paxinos, G., and Franklin, K.B.J. (2008). The Mouse Brain in Stereotaxic Coordinates (Gulf Professional Publishing).

Phelps, E.A., and LeDoux, J.E. (2005). Contributions of the Amygdala to Emotion Processing: From Animal Models to Human Behavior. Neuron *48*, 175–187.

Pryce, C.R., and Fontana, A. (2016). Depression in autoimmune diseases. Curr. Top. Behav. Neurosci., in press.

Pryce, C.R., Azzinnari, D., Sigrist, H., Gschwind, T., Lesch, K.-P., and Seifritz, E. (2012). Establishing a learned-helplessness effect paradigm in C57BL/6 mice: Behavioural evidence for emotional, motivational and cognitive effects of aversive uncontrollability per se. Neuropharmacology *62*, 358–372.

Pugh, C.R., Kumagawa, K., Fleshner, M., Watkins, L.R., Maier, S.F., and Rudy, J.W. (1998). Selective Effects of Peripheral Lipopolysaccharide Administration on Contextual and Auditory-Cue Fear Conditioning. Brain. Behav. Immun. *12*, 212–229.

Qin, L., Wu, X., Block, M.L., Liu, Y., Breese, G.R., Hong, J.-S., Knapp, D.J., and Crews, F.T. (2007). Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration. Glia *55*, 453–462.

Raison, C.L., Rutherford, R.E., Woolwine, B.J., Shuo, C., Schettler, P., Drake, D.F., Haroon, E., and Miller, A.H. (2013). A randomized controlled trial of the tumor necrosis factor antagonist infliximab for treatment-resistant depression: the role of baseline inflammatory biomarkers. JAMA Psychiatry *70*, 31–41.

Salazar, A., Gonzalez-Rivera, B.L., Redus, L., Parrott, J.M., and O'Connor, J.C. (2012). Indoleamine 2,3-dioxygenase mediates anhedonia and anxiety-like behaviors caused by peripheral lipopolysaccharide immune challenge. Horm. Behav. *62*, 202–209.

Silverman, M.N., Macdougall, M.G., Hu, F., Pace, T.W.W., Raison, C.L., and Miller, A.H. (2007). Endogenous glucocorticoids protect against TNF-alpha-induced increases in anxiety-like behavior in virally infected mice. Mol. Psychiatry *12*, 408–417.

Smith, R.S. (1991). The macrophage theory of depression. Med. Hypotheses *35*, 298–306.

Thomson, L.M., and Sutherland, R.J. (2005). Systemic administration of lipopolysaccharide and interleukin-1β have different effects on memory consolidation. Brain Res. Bull. *67*, 24–29.

Ueno, M., Akiguchi, I., Hosokawa, M., Kotani, H., Kanenishi, K., and Sakamoto, H. (2000). Blood-brain barrier permeability in the periventricular areas of the normal mouse brain. Acta Neuropathol. (Berl.) *99*, 385–392.

Wang, C., Wang, C.-M., Clark, K.R., and Sferra, T.J. (2003). Recombinant AAV serotype 1 transduction efficiency and tropism in the murine brain. Gene Ther. *10*, 1528–1534.

Warden, D., Rush, A.J., Trivedi, M.H., Fava, M., and Wisniewski, S.R. (2007). The STAR*D Project results: a comprehensive review of findings. Curr. Psychiatry Rep. *9*, 449–459.

Willner, P. (1997). Validity, reliability and utility of the chronic mild stress model of depression: a 10-year review and evaluation. Psychopharmacology (Berl.) *134*, 319–329.

Wohleb, E.S., Powell, N.D., Godbout, J.P., and Sheridan, J.F. (2013). Stress-Induced Recruitment of Bone Marrow-Derived Monocytes to the Brain Promotes Anxiety-Like Behavior. J. Neurosci. *33*, 13820–13833.

World Health Organization (1992). The ICD-10 Classification of Mental and Behavioural Disorders: Clinical Descriptions and Diagnostic Guidelines (Geneva: World Health Organization).

Zolotukhin, S., Byrne, B.J., Mason, E., Zolotukhin, I., Potter, M., Chesnut, K., Summerford, C., Samulski, R.J., and Muzyczka, N. (1999). Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield. Gene Ther. *6*, 973–985.

Figure legends

Figure 1. A-D. Effects of 10-day daily i.p. murine TNF (40 ng/g) versus vehicle on cytokine levels and body weight. A) Day 9 plasma TNF levels at 1, 3 and 6 hours post-injection. B) Day 10 plasma IL-6 and IL-10 levels at 1 h post-injection of TNF (n=4 per group), nd: not detectable. C) Daily body weight relative to day 0 baseline. D) Day 10 TNF tissue level in amygdala, periventricular regions and medial hippocampus, in brains collected at 1 h post-injection. E) Effects of 5-day daily i.p. human TNF injection at 40 ng/g (low hTNF40) versus VEH or single i.p. human TNF injection at 400 ng/g (high hTNF) on brain tissue levels of human TNF protein at 1 hour post-injection, in periventricular regions, medial hippocampus and amygdala. In A) and C), values are mean +/- SD. In A), ** $p<0.01$, *** $p<0.001$ in post hoc LSD test. In C) ** $p<0.01$ for main effect of group. In D) p value is for unpaired t-test. Dashed line indicates assay lower limit of detection.

Figure 2. Effects of 4-day daily i.p. murine TNF (20 ng/g, 40 ng/g) versus vehicle in auditory CS fear conditioning and memory expression tests conducted at days 4 and 5, respectively. A) CS-US fear conditioning on day 4, as % time freezing during pairs of consecutive CS trials. B) CS fear expression on day 5, as % time freezing during pairs of consecutive CS trials. Values are mean +/- SD. A group X trial linear mixed model was conducted and significant effects further analyzed by post hoc LSD test. In A) trials indicated by different letters are different at $p<0.05$ or lower. In B) * $p<0.05$ for main effect of group, and LSD test identified increased freezing by IP-TNF40 mice relative to both other groups.

Figure 3. Effects of 6 days (days 1-6) daily i.p. injection of murine TNF (20 ng/g, 40 ng/g) versus vehicle on saccharin consumption (g) in the two-bottle test on days 4-9. Day -1 was the final day of baseline data collection. A) Group comparison for saccharin consumption. Group x Day analysis for days 4-9 yielded an interaction that justified a posteriori group-specific analysis of saccharin consumption relative to baseline. B) IP-VEH mice. C) IP-TNF20 mice. D) IP-TNF40 mice. Values are mean +/- SD. In A) * denotes $p<0.05$ for IP-TNF40 versus VEH mice following group X day interaction effect and post hoc LSD test. In B)-D) ** $p<0.01$, *** $p<0.001$ versus day -1 baseline following day main effect and post hoc LSD test. Black bar indicates final 3 days of i.p. injection.

Figure 4. Effects of subcutaneous delivery of murine TNF (40 µg/mL) or vehicle by osmotic micropump on days 1-7 on TNF levels and body weight and, in a separate cohort, saccharin drinking. A) TNF plasma levels on days 1, 3 and 6 after implantation (day 0). B) Daily body weight relative to day 0 baseline prior to pump implantation. C) Group comparison for saccharin consumption. Group x day anaylsis for days 1-5 yielded an interaction that justified a posteriori group-specific analysis of saccharin consumption relative to baseline. D) OP-VEH mice. E) OP-TNF40 mice. Values are mean +/- SD. In A), B) and C), * $p<0.05$, ** $p<0.01$, *** $p<0.001$ for OP-TNF40 versus OP-VEH mice following group X day interaction effect and post hoc LSD test. In E), ** $p<0.01$, *** $p<0.001$ versus day -3 baseline following day main effect and post hoc LSD test. Dashed lines in A) indicates assay LLOQ, and in B) indicates body weight on day 0.

Figure 5. Local effects of region-specific injection and 14-day expression of a scrAAV-8/2-CMV-chI-murine TNF vector (AAV-TNF), control vector (AAV-CON), or vehicle (VEH). A)

Hippocampal tissue TNF concentration following hippocampal injection. B) Percent area of amygdala expressing Iba-1 as a marker for microglia activation following amygdala injection. In A), dashed line depicts assay LLOQ; nd: not detectable. In B) *** $p<0.001$ for group main effect followed by post hoc LSD test. Representative sections depicting amygdala region from C) AAV-CON mouse and D) AAV-TNF mouse.

Figure 6. Effects of bilateral i.c.v. injection of AAV-TNF (1.1e11 vg/ml) versus AAV-CON (1.1e10 vg/ml). A) Day 22 mouse-specific TNF concentration in tissue from periventricular regions. TNF levels in AAV-CON mice were below assay LLOQ (0.3 pg/ml; this value was assigned for analysis). Dashed line represents limit of detection based on assay LLOQ and average protein concentration. nd: not detectable. B) Mean-weekly body weight relative to day 0 baseline. C) Days 20-22 saccharin consumption in the two-bottle test. D-E) Days 14-15 contextual fear conditioning and memory expression tests: D) Day 14 % time freezing during intervals between footshock US (ITIs). E) Day 15 % time freezing during 1-min intervals. F-H) Day 16 two-way avoid-escape test comprising 30 CS-US trials analyzed in 3 x 10-trial blocks: F) Avoid-escape failures. G) Per cent time freezing. H) ITI compartment transfers. Values are mean +/- SD. In A), * $p< 0.05$ in unpaired t-test. In B)-H), * $p<0.05$, ** $p<0.01$ for group main effect or interaction effect followed by post hoc LSD test. Data points indicated by different letters are significantly different at $p<0.05$ or lower for trial main effect followed by post hoc LSD test.

Figure 7. Effects of bilateral hippocampal injection of AAV-TNF (1.1e10 vg/ml) versus AAV-CON (1.1e10 vg/ml). A) Day 16 mouse-specific TNF concentration in hippocampal tissue. TNF levels in AAV-CON mice were below assay LLOQ. Dashed line represents limit of detection;

nd: not detectable. B) Mean-weekly body weight relative to day 0 baseline. C-D) Days 14-15 contextual fear conditioning and memory expression tests: C) Day 14 % time freezing during intervals between footshock US (ITIs). D) Day 15 % time freezing during 1-min intervals. E) Memory consolidation index comparing % time freezing during expression intervals 1-2 (see C) with % time freezing during conditioning trials 5-6 (see D). F-H) Day 16 two-way avoid-escape test comprising 30 CS-US trials analyzed in 3 x 10-tral blocks: F) Avoid-escape failures. G) Per cent time freezing. H) ITI compartment transfers. Values are mean +/- SD. In A) * $p<0.05$ in unpaired t-test. In E) $p<0.05$ in paired t-test. Otherwise, * $p<0.05$, ** $p<0.01$, *** $p<0.001$ for group main effect or interaction effect followed by post hoc LSD test. Data points indicated by different letters are significantly different at $p<0.05$ or lower for trial main effect followed by post hoc LSD test.

Figure 8. Effects of bilateral amygdala injection of AAV-TNF (1.1e10 vg/ml) versus AAV-CON (1.1e10 vg/ml). A) Day 22 mouse-specific TNF concentration in amygdala tissue. TNF levels in AAV-CON mice were below assay LLOQ. Dashed line represents limit of detection; nd: not detectable. B) Mean-weekly body weight relative to day 0 baseline. C) Days 20-22 saccharin consumption in the two-bottle test. D-E): Days 14-15 CS fear conditioning and memory expression tests: D) Day 14 % time freezing during CS of CS-US trials. E) Day 15 % time time freezing during CS trials. F-H) Day 16 two-way escape test comprising 30 US trials analyzed in 3 x 10-trial blocks: F) Escape failures. G) Per cent time freezing. H) ITI compartment transfers. Values are mean +/- SD. In A) *** $p<0.001$ in unpaired t-test. In B-H), * $p<0.05$, ** $p<0.01$ for group main or interaction effect followed by post hoc LSD test. Data points indicated by different letters are significantly different at $p<0.05$ or lower for trial main effect followed by post hoc LSD test.